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Effects of ammonia and climatic parameters on the welfare of sheep during live export, including the development of a sampling strategy for environmental monitoring on ships

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MSc., BSc.

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School of Veterinary Science

Abstract

Mortality is the most important indicator for welfare assessment of livestock exported from Australia to the Middle East by sea. Relevant research on the causes of sheep death during sea voyages is limited. In the early years of live export 43% were attributed to inanition, associated with inadequate fat mobilization for energy supply when the sheep are inappetant on board shipments. However, little is known about the influence of the micro-climatic conditions (in particular ammonia accumulation) on ships and the climatic conditions before embarkation.

Ammonia accumulation in sheep pens, as a top welfare issue, adversely affects sheep feed intake, which is related to a cause of mortality, in the form of inanition. However, little is known about the mechanisms underlying this effect. A first experiment investigated the nutritional behaviour and stress levels in sheep exposed to ammonia at a similar concentration to those experienced by sheep during sea export. Twelve sheep were randomly allocated to Ammonia (30 ppm, 21 mg/m^3) or Control treatment in a changeover design with three 2-week periods. Ammonia exposure reduced feed intake and defecation time and slowed the rates of eating hay, masticating alfalfa pellets and rumination chewing. It also lengthened the pause between swallowing and regurgitation during rumination, increased faecal corticosterone metabolites concentration on day 6, increased respiratory rate, and reduced yawning. The increase in faecal corticosterone metabolites concentration in ammonia exposed sheep was not correlated with the reduction in feed intake. The results suggest that although sheep exposed to ammonia typical of a live export shipment are stressed, this is not the reason for reduction in feed intake. They may have an irritation in the buccal cavity, which retards nutritional behaviour, and causes shallow rapid breathing to minimise irritation to the lungs.

Climatic factors are also implicated in sheep mortality on sea voyages from Australia to the Middle East. The second study explored the relationships between sheep mortality and climatic conditions at departure and destination ports as well as the type of voyage. Mortality data from 417 shipments of sheep exported over an 11-year period (November 2004 to June 2015) were modelled retrospectively to determine the key correlates. Statistical analysis was performed for both a full dataset with 417 voyages based on actual and estimated departure and arrival dates and a restricted dataset with 71 voyages based on actual dates. Results of the full dataset demonstrate a seasonal mortality pattern, with higher sheep mortality rate occurring on sea voyages leaving Australia in the southern hemisphere winter or spring than those departing in Australian summer or autumn. Heat stress and inadequate fat mobilization for energy supply when sheep are inappetant on shipments may explain this seasonality. Based on two models, the voyage and weather factors associated with

sheep mortalities include departure year, autumn departure season in the southern hemisphere, voyage duration, single or multiple loading port(s), weekly mean dry bulb temperature and mean wind speed at the departure ports, and weekly mean humidity at the destination ports. Significant correlations were observed between weather variables at the departure ports in the Australian winter and a high sheep mortality rate. This, together with the heat stress risk at this time, which is expected to increase as a result of climate change, suggest that there could be review of the trade from Australia in the southern hemisphere winter. The influence of weather at the departure ports should be considered in sheep mortality prediction models, such as the Australian Heat Stress Risk Assessment model.

The adverse welfare impacts and risks linked to the ammonia accumulation and climatic parameters highlight the necessity of environmental monitoring on board shipments during sheep export from Australia to the Middle East. However, none of these parameters has yet been effectively measured currently on ships. The third study developed an effective sampling strategy for the measurement of micro-climatic conditions during live export from an analysis of measurement uncertainty. Based on the data maps previously obtained on two typical voyages of sheep export, optimum sampling strategies for ammonia and temperature measurement on the vessel were determined. The difference between predicted High and Low ammonia sites on these shipments was accurately detected with five sampling sites of each. Margins of error were determined, which suggested that dry bulb temperature could be accurately measured on ships with six to eight sampling sites, but even twenty sampling sites were not sufficient to accurately measure relative humidity. For the vessel recorded, considerably more sampling sites are required for ammonia measurement on closed decks than on open decks, as a result of greater variation in the former, but there was less variation for temperature measurement. The number of pens measured contributed more to the variance of ammonia and temperature measurement than the number of sampling sites within each pen on open decks.

This research project highlights the seasonal sheep mortality pattern and the importance of more detailed monitoring and effective management of sheep welfare during live export by sea from Australia to the Middle East.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, financial support and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my higher degree by research candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Peer-reviewed papers

Zhang Y and Phillips CJC 2018 Climatic influences on the mortality of sheep during long-distance sea transport. *Animal*. In press

Zhang Y, Guinnefollau L, Sullivan M and Phillips CJC 2018 Behaviour and physiology of sheep exposed to ammonia at a similar concentration to those experienced by sheep during export by sea. *Applied Animal Behaviour Science* 205:34-43

Zhang Y, Lisle A and Phillips CJC 2017 Development of an effective sampling strategy for ammonia, temperature and relative humidity measurement during sheep transport by ship. *Biosystems Engineering 155:*12-23

Book chapters

Zhang Y and Phillips CJC 2018. The effects of atmospheric ammonia during export of livestock. In: Banhazi T, Aland A and Hartung J (eds.) *Air and environmental quality in livestock/agricultural buildings* pp 193-204. CRC Press: Florida, USA.

Conference abstracts

Zhang Y, Pines M and Phillips CJC 2015 Ammonia on a live export shipment, effects on sheep behaviour and development of an effective ammonia sampling strategy. In: *Proceedings of the 49th Congress of the International Society for Applied Ethology.* pp 74. Sapporo, Japan.

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Scientific meetings

Zhang Y 2014 *48th Congress of the International Society for Applied Ethology - Australasia and Africa Regional Meeting*: October 29th Sydney, NSW, Australia.

Zhang Y 2015 Ammonia on a live export shipment, effects on sheep behaviour and development of an effective ammonia sampling strategy. *49th Congress of the International Society for Applied Ethology*: September 14th -16th Sapporo Hokkaido, Sapporo, Japan.

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No manuscripts included.

Contributions by others to the thesis

The concept for, and design of, this research, and interpretation of results were achieved through the active collaboration of me and my principal supervisor Professor Clive Phillips. The design of the methodology of Chapter 3 & 4 research was produced through discussions and consultations with the Statistical Consultant Allan Lisle. The enzyme immunoassay procedure required for faecal corticosterone metabolites measurement in Chapter 2 was guided by Dr Tamara Keeley in the training, who also helped complete faecal cortisol metabolites measurement. The personnel of the Queensland Animal Science Precinct (QASP) in The University of Queensland were actively involved in the technical aspects necessary for the sheep husbandry in Chapter 2 research.

Statement of parts of the thesis submitted to qualify for the award of another degree

Part of the description of the materials, methodology and results detailed in Chapter 2 was used in the thesis of Miss Lauréline Guinnefollau to obtain her Master's Degree on Behavioural Ecology, Evolution and Biodiversity from the University François Rabelais (Tours - France), 2015, degree awarded on September 2015. The experiment design, some results of feed palatability test, feed intake measurement, some results of ruminating behaviour observation, and cognitive bias training and test were included in her thesis, recognising that she helped in this work. Her analysis of the raw data obtained from the cognitive bias test was done independently from this work, for presentation in her thesis. The description of the experimental methodology, statistical analysis, and the final discussion and conclusion from the work included in her thesis was modified and improved for presentation in this PhD thesis.

Research involving human or animal subjects

No human subjects were involved in this research. The animal experiment included in Chapter 2 research was approved by the Production and Companion Animals Ethics Committee of The University of Queensland (CAWE/242/14, Appendix-1).

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Keywords

ammonia, behaviour, climate, environmental monitoring, feed intake, mortality, sampling strategy, season, sheep welfare, transport

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ANZSRC code: 070203, Animal Management, 50%

Fields of Research (FoR) Classification

FoR code: 0707, Veterinary Sciences, 60%

FoR code: 0702, Animal production, 40%

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List of Abbreviations used in the thesis

ACGIH = American Conference of Governmental Industrial Hygienists

ADF = Acid Detergent Fibre

AIC = Akaike Information Criterion

AIHA = American Industrial Hygiene Association

AN = Ambiguous, partially Negative Location

ANOVA = Analysis of Variance

AP = Ambiguous, partially Positive Location

ASEL = Australian Standards for the Export of Livestock

BAL = Bronchoalveolar Lavages

BW = Body Weight

CB = Cognitive Bias

CI = Confidence Interval

Coef = Coefficient

CC = Correlation Coefficient

CP = Crude Protein

D = day

df = Degree of Freedom

DMI = Dry Matter Intake

DP = Dew Point

DP_m = Mean Dew Point

ERPG = Emergency Response Planning Guidelines

FCE = Feed Conversion Efficiency

FCM = Faecal Cortisol Metabolites

FCSM = Faecal Corticosterone Metabolites

GLM = General Linear Model

h = hour

H_m = Mean Humidity

HSRA = Heat Stress Risk Assessment

M = Middle Ambiguous Location

ME = Metabolisable Energy

min = minute

MLA = Meat and Livestock Australia

MoE = Margin of Error

N = Negative Location

NDF = Neutral Detergent Fibre

NEFA = Non-esterified Fatty Acid

NH_3 = ammonia

NIOSH = National Institute for Occupational Safety and Health

NOHSC = National Occupational Health and Safety Commission

OSHA = Occupational Safety and Health Administration

P = Positive Location

PEL = Permissible exposure limit

ppm = parts per million

QASP = Queensland Animal Science Precinct

RH = Relative Humidity

RR = Respiratory Rate

RSPCA = Royal Society for the Prevention of Cruelty to Animals

RT = Rectal Temperature

SA = South Australia

SD = Standard Deviation

sec = second

SED = Standard Error of the Difference

SEM = Standard Error of the Mean

TAS = Tasmania

T_{DB} = Dry Bulb Temperature

T_{DBm} = Mean Dry Bulb Temperature

TDN = Total Digestible Nutrients

THI = Temperature Humidity Index

TN = Northern Territory

TWA = Time Weighted Average

T_{WB} = Wet Bulb Temperature

T_{WBm} = Mean Wet Bulb Temperature

UEP = United Egg Producers

USEPA = US Environmental Protect Agency

VIC = Victoria

VIF = Variance Inflation Factor

WA = Western Australia

WDT = Weather Decision Technologies

WS_m = Mean Wind Speed

1 Chapter 1: General introduction

1.1 Effects of atmospheric ammonia on livestock during export

Poor air quality on live export shipments can adversely affect the health and performance of animals, as well as those looking after them. One major contributor is ammonia (NH_3) (MLA 2001; Tudor, *et al.* 2003; McCarthy 2005), which is a colorless, highly irritating alkaline gas. Although there is limited evidence for the effects of NH_3 on livestock exported, many studies have been conducted in pigs and poultry under intensive housing conditions (Stombaugh, *et al*. 1969; Drummond, *et al*. 1980; Urbain, *et al*. 1994, 1996, Jones & Webster 1998; Jones, *et al*. 1996, 2000, 2001; David, *et al*. 2015; Michiels, *et al*. 2015). Ammonia accumulation has been identified as a top welfare issue related to the sea transport of livestock by Australian veterinarians, livestock exporters, and ship owners (Pines, *et al.* 2007), in particular when the levels exceed the 8-hour time-weighted average (TWA) exposure level recommended for humans (25 ppm, 17 mg/m^3).

1.1.1 Source of atmospheric ammonia during live export

As in other intensive animal housing systems, the conditions onboard live export vessels are conducive to the NH_3 accumulation, due to the high stocking densities of animals and feces and urine accumulation. For cattle export, the waste is cleaned out regularly, such as every 3 days. However, for sheep export, as long as humidity is low, a pad of soft, friable material is formed and will not be cleaned out until the animals have been discharged at the end of the voyage, usually after 10-26 days (Phillips 2008). Ammonia is generated by bacterial urease enzymes in bedding or manure pads acting on urea in urine and undigested protein in faeces (Costa, *et al.* 2003). Unlike poultry, which excrete uric acid and undigested proteins in their faeces, cattle, sheep and pigs excrete superfluous nitrogen as urea in the urine and undigested proteins in the faeces (Groot Koerkamp, *et al.* 1998). Ambient temperature, urine concentration and pH levels of the pad all contributed to the NH_3 accumulation during live export (Costa, *et al.* 2003).

1.1.2 Atmospheric ammonia levels during live export

One study has shown that typical NH_3 levels measured in vessels transporting cattle and sheep from Australia to the Middle East were 15 ppm (10 mg/m^3), with most common readings of between 20 to 30 ppm (14 to 21 mg/m^3) (MLA 2001). Pines & Phillips (2011) showed the levels at most of their measurement locations for similar voyages were below 18 ppm (13 mg/m^3). However, the mean level at several sites was above 25 ppm (17 mg/m^3), due to insufficient ventilation and/or high temperatures and humidity. The high level at some sites achieved to 59 ppm (41 mg/m^3), which is above the permissible exposure limit (PEL) (50 ppm, 35 mg/m^3) recommended for humans by the

National Occupational Health and Safety Commission (NOHSC 1995). So far, there is no universally applied maximum NH_3 concentration for live export shipments. Some for the Australian live export industry (MLA 2001; Costa, *et al.* 2003) recommend a level of 25 ppm (17 mg/m^3), which is the 8-hour TWA exposure level also suggested for humans. However, since a TWA level does not apply to livestock on ships, a physiologically-validated maximum NH_3 concentration of 30 ppm (21 mg/m^3) has been proposed for steers (Phillips, *et al.* 2010), with supporting evidence that this may be appropriate for sheep (Phillips, *et al.* 2012a).

During livestock export, the major factors influencing atmospheric NH_3 levels include temperature, humidity, ventilation efficiency, stocking density, prior diet, shipboard diet, manure characteristics (depth and moisture content of the faecal pad), and deck washing on ships (Costa, *et al.* 2003). Ammonia concentrations on board shipments may be increased by high humidity and air temperatures encountered while vessels travel through tropical and equatorial regions. Another important contributing factor is likely to be ventilation inadequacy. Limited measurements of NH_3 accumulation on a live export voyage from Australia to the Middle East suggest that NH_3 accumulates quickly, while a gradual rise over the first 10 days was discernible on closed decks. On open decks, there was initially a rapid accumulation, then a reduction due to ventilation on the open sea, and a final increase in the more sheltered conditions of the Persian Gulf in the last few days. The NH_3 levels were consistently higher on closed decks than on open decks (Pines & Phillips 2011). Correlations were found between NH_3 concentrations on the closed decks and dew point, as well as wet and dry bulb temperature, while on the open decks the correlations were with cumulative wind during the voyage, air speed, dew point, wet bulb temperature and faecal pad depth, (Pines & Phillips 2011).

1.1.3 Effects of ammonia on livestock during live export

Ammonia exposure has been shown to be detrimental to the health and performance of animals. Most research on the effects of NH_3 focused on pigs and poultry. However, some studies have also been documented in sheep and cattle, as well as in simulated sea export from Australia.

1.1.3.1 Effects of ammonia on growth and production performance

Production indices adversely affected by NH_3 exposure in different animal species include feed intake, average daily weight gain and feed conversion efficiency (FCE). Under a simulated ship journey conditions, although no effect of NH_3 on body weight (BW) was observed in steers, feed intakes of sheep were decreased in proportion to NH_3 concentrations between 15 and 45 ppm (10 and 31 mg/m^3), and at high concentrations, BW gains were decreased (Phillips, *et al.* 2010, 2012a).

A concentration of 45 ppm (31 mg/m^3) in this study is close to the maximum mean value (50 ppm, 35 mg/m^3) in any environment that humans can legally enter (NOHSC 1995). Reduced FCE and BW gain were also demonstrated in sheep exposed to 75 ppm (52 mg/m^3) NH_3 for 28 days (Drummond, *et al.* 1976).

Similar effects of NH_3 exposure on growth and production have been extensively reported in poultry and pigs. In broiler chickens during the 0-3 week period, exposure to 52 ppm (36 mg/m^3) NH_3 reduced BWs and feed to gain ratios (Wang, *et al.* 2010). Likewise, reduced BWs and feed intakes in proportion to NH_3 concentrations between 16 and 54 ppm (11 to 38 mg/m^3) were observed in broiler chickens during the 4-7 week period (Yahav 2004). Continuous exposure to 25 and 50 ppm (17 and 35 mg/m^3) NH_3 during a 4-week brooding period reduced birds' BWs at 7 weeks of age. This was also the consequence of exposure to 50-75 ppm (35 to 52 mg/m^3), which also increased mortality, even though no reported influence on FCE (Reece & Lott 1980, 1981; Miles, *et al.* 2004). Similar responses were found in layer hens subjected to high NH_3 in enclosed sheds. Exposure to 78 ppm (54 mg/m^3) NH_3 reduced feed intakes and growth rates, resulting in delayed maturation of replacement laying pullets (Charles & Payne 1966a, 1966b). Exposing 12-month-old laying hens or pullets at point of lay to 200 ppm (139 mg/m^3) NH_3 for 17 days reduced egg production, BWs and feed intakes, and increased post-exposure mortality in pullets (Deaton, *et al.* 1982, 1984).

In contrast to little or no observable effect on growth and production of pigs at, or slightly above, commercially realistic NH_3 concentration (20 ppm, 14 mg/m^3) (Curtis, *et al.* 1975; Wathes, *et al.* 2004; Von Borell, *et al.* 2007; Cheng, *et al.* 2014), detrimental effects of NH_3 were evident in pigs exposed to higher NH_3 concentrations. A concentration-related reduction in weight gain were reported in pigs exposed to 25, 50, 100 and 150 ppm (17, 35, 70, 104 mg/m^3) NH_3 (Drummond, *et al.* 1980; Urbain, *et al.* 1994). Compared to 12 or 61 ppm (8 or 42 mg/m^3) NH_3 treatment, exposure to 103 or 145 ppm (72 or 101 mg/m^3) NH_3 reduced feed consumption and average daily weight gains of pigs, but there was no effect on FCE (Stombaugh, *et al.* 1969). Besides, exposure to the combination of airborne pollutants including ammonia (13 ppm, 9 mg/m^3), carbon dioxide and dust reduced weaner pigs' feed intake and growth rates from 3 to 8 weeks of age (Lee, *et al.* 2005). Nutrients were redirected away from production and towards the immune system in pigs at 16 weeks of age after exposure to α-haemolytic cocci in the presence of ammonia at concentrations of 10, 25 and 50 ppm (17, 35 and 52 mg/m^3) (Murphy, *et al.* 2012).

1.1.3.2 Effects of ammonia on health

As a water-soluble irritant, NH_3 can easily dissolve in moisture in the air, and on tissue or mucous membranes, to form ammonium hydroxide and cause inflammation of the mucous membrane in the eyes and the respiratory tract. In humans, Pritchard (2007) concluded that acute inhalation of NH_3 may initially cause upper respiratory tract irritation. Substantial exposure can cause burns in the oral cavity, nasopharynx, larynx and trachea, together with airway obstruction, respiratory distress and bronchiolar and alveolar oedema. Chronic inhalation has been associated with increased coughing, mucus production, wheezing and asthma. Therefore, NH_3 is considered as a risk factor causing lung disease in humans, in particular agricultural workers (Omland 2002).

Respiratory dysfunction. Although the anatomical structure of the respiratory system differs between animal species, respiratory dysfunction is the most frequently observed sign of NH_3 exposure. Respiratory system defenses against inhaled pollutants, by integrating complex biochemical, physiological and immunological processes, which depend on the properties of the inhaled particles (Korpas & Tomori 1979).

In simulated ship journeys, NH_3 exposure resulted in a transitory adverse effect on the livestock health. Exposure to 22 and 42 ppm (15 and 29 mg/m^3) NH_3 in simulation rooms for 9 days increased total white cell and mononucleated cell counts of the Bronchoalveolar Lavages (BAL) samples in Angus-cross heifers (Accioly, *et al.* 2004). Further evidence of active pulmonary inflammation derives from increased macrophage activity in BAL samples from steers and transtracheal aspirations from sheep (Phillips, *et al.* 2010, 2012a). In these studies, 30 and 45 ppm (21 and 31 mg/m^3) NH_3 increased BAL neutrophils in steers, which is consistent with previous research in pigs showing that neutrophil count is positively and linearly related to NH_3 concentrations (25, 50, and 100 ppm; 17, 35 and 70 mg/m^3) in nasal lavages (Urbain, *et al.* 1994). Moreover, increased frequency of sneezing in sheep and increased lacrimation, nasal secretions and coughing in steers indicate the irritation of the mucous membranes of eyes, nasal cavity and respiratory tract. Exposure to 75 ppm (52 mg/m^3) NH_3 for 28 days also adversely affected respiratory function in lambs, with severe coughing and sneezing, profuse lacrimation and nasal discharge, which contained blood in some instances (Drummond, *et al.* 1976). However, it is unknown to what extent such high levels occur during sheep transport and if they do, whether respiratory function is impaired in this way.

Similar effects of NH_3 exposure have been documented in poultry and pigs. Chronic exposure to 20 ppm (14 mg/m^3) NH_3 adversely affected hens' respiratory health, and caused gross or

histopathological signs of damage to the respiratory tract in chickens and turkeys (Anderson, *et al.* 1964; Sales 2012). Exposure to 25 and 50 ppm (17 and 35 mg/m^3) NH_3 for 8 weeks caused severe airsacculitis in broilers at 6 and 8 weeks of age, with increased airborne bacteria (Quarles & Kling 1974). Exposure to 60-70 ppm (42-49 mg/m^3) NH_3 can result in tracheitis due to irritation of the mucous membrane (Valentine 1964). Even short exposure to increasing NH_3 concentrations (50, 75, or 100 ppm; 35, 52 or 70 mg/m^3) for 4 days led to an increase in thickness of the atrial walls and a shrinking of air capillaries in broilers, particularly at the two higher levels (Al-Mashhadani & Beck 1985).

In pigs, the synergistic role of NH_3 in facilitating the growth and/or survival of *P. multocida* within the upper respiratory tract was highlighted, which contributed to the severity of the atrophic rhinitis (Hamilton, *et al.* 1996). A 6-day exposure to 25 ppm (17 mg/m^3) NH_3 caused nasal irritation and functional disturbances of the tracheal smooth-muscle contractions in pigs (Urbain, *et al.* 1996). As little as a 15-minute exposure to 10, 25 and 50 ppm (17, 35 and 52 mg/m^3) NH_3 caused a mild to severe alveolitis in proportion to NH_3 concentrations (Murphy, *et al.* 2012). Prolonged exposure to as low as 9 ppm (6 mg/m^3) NH_3 also had a detrimental effect on development of the nasal turbinates in the absence of bacterial flora in young growing pigs (Jones & Webster 1998). Exposure to levels of NH_3 above 50 ppm (35 mg/m^3) resulted in a mild to heavy acute exudate in the turbinate lumen of some pigs, and an acute inflammatory reaction in the tracheal epithelium (Drummond, *et al.* 1980).

Ocular disease. Keratoconjunctivitis, known as an inflammation of the cornea and conjunctiva, has been described several times in association with NH_3 exposure. In a simulated ship journey, increased lacrimation in steers indicated that the irritation occurred in the mucous membranes of the eyes, particularly at a level of 45 ppm (31 mg/m^3) (Phillips, *et al.* 2010); this has been confirmed on an actual ship journey in sheep (Pines & Phillips, 2013). Similarly, NH_3 also caused keratoconjunctivitis in chicks' eyes (Faddoul & Ringrose 1950). Chronic exposure to 20 ppm (14 mg/m^3) NH_3 could adversely affect hens' ocular health (Sales 2012), while 30 and 60 ppm (21 and 42 mg/m^3) exposure increased conjunctival lesions in broiler chicks reared to 21 days (Beker, *et al.* 2004). The speed of recovery from NH_3-induced keratoconjunctivitis depends on the severity of the ulcers (Valentine 1964). In broiler chickens exposed to 25, 50 and 75 ppm (17, 35 and 52 mg/m^3) NH_3, ocular abnormalities appeared, with more severe lesions observed at higher concentrations. When aerial NH_3 diminished after 28 days of the grow-out stage, eye health improved, especially uveitis, even though lymphocytes and heterophils could be seen in the iris up to 49 days later in NH_3-exposed birds (Miles, *et al.* 2006). Further evidence of rapid recovery from uveitis was found

after exposing broiler chickens to 25 and 50 ppm (17 and 35 mg/m^3) NH_3 for 7 days (Olanrewaju, *et al.* 2007).

Susceptibility to disease. Inflammation responses in cattle and sheep exposed to NH_3 during live export may make them more susceptible to disease, as has been observed in poultry and pigs. Broiler chickens exposed to 26 and 52 ppm (18 and 36 mg/m^3) NH_3 had lower Newcastle disease virus hemaglutination inhibition antibody titers during the first 3 weeks, compared to those in a 13 ppm (9 mg/m^3) treatment or a control group (Wang, *et al.* 2010). Either 72-hour exposure to 20 ppm (14 mg/m^3) NH_3 or 48-hour exposure to 50 ppm (35 mg/m^3) increased infection rates, both clinically and serologically, when the chickens were subsequently exposed to an aerosol of Newcastle disease virus (Anderson, *et al.* 1964). Similarly, after Leghorn males were vaccinated for infectious bronchitis, exposure to 25 and 50 ppm (17 and 35 mg/m^3) NH_3 from 4 to 8 weeks of age reduced the weight of bursa of fabricius, a part of the avian immune system (Kling & Quarles 1974). It was suggested that maybe a more severe reaction to the vaccine caused by NH_3 stress elicited a greater response from the bursae. In turkeys, more *Escherichia coli* was found in the lungs, air sacs and livers after exposure to 10 and 40 ppm (7 and 28 mg/m^3) NH_3, compared to the control group, which had better clearance of *Escherichia coli* (Nagaraja, *et al.* 1984). Similar effects were observed in pigs exposed to 50 and 75 ppm (35 and 52 mg/m^3) NH_3, in which 50 ppm (35 mg/m^3) NH_3 also increased pulmonic weight and the ratio of pulmonic weight to body weight, compared to the controls (Drummond, *et al.* 1978).

Other physiological parameters. In simulated ship journeys, NH_3 exposure had no effect on a limited number of hematological variables in steers and sheep (Phillips, *et al.* 2010, 2012a). However, changes were found in some other physiological parameters in pigs and poultry, which may be explored in further research during live export of cattle and sheep.

The distribution of NH_3 between body compartments is influenced by blood pH, with the transfer of NH_3 being dependent upon arterial pH and systemic alkalosis exacerbating NH_3 toxicity (Ott & Larsen 2004). In broiler chickens, exposure to 25-75 ppm (17-52 mg/m^3) NH_3 increased the partial pressure of CO_2 and blood pH, hematocrit and hemoglobin, reduced partial pressure of O_2, bicarbonate and K^+, but had no influence on plasma corticosterone concentrations. An interaction between NH_3 and age was observed for blood pH, anion gap and HCO_3^-, with younger birds having a more intense reaction than older birds if judged from the physiological responses (Olanrewaju, *et al.* 2009). In Yahav's (2004) study, arterial pH tended to increase with NH_3 concentrations (16, 28, 39 and 54 ppm; 11, 20, 27 and 38 mg/m^3), but there was no difference between the two highest

levels, while the partial pressure of arterial CO_2 showed a similar but opposite trend, but there was no influence on plasma T3 concentration. Ammonia may also affect broiler chickens' ability to control body temperature, as this was more effectively regulated at 16 and 28 ppm (11 and 20 mg/m^3) compared with at 39 and 54 ppm (27 and 38 mg/m^3) (Yahav 2004). The plasma NH_3 concentration of broilers increased with NH_3 exposure levels (13, 26, or 52 ppm (9, 18, or 36 mg/m^3) from 0 to 3 weeks of age; 20, 40, or 80 ppm (14, 28, or 56 mg/m^3) from 4 to 5 weeks, but there was no influence on plasma uric acid (Song, *et al.* 2008). In a simulated ship journey, some prolonged but inconclusive effects of NH_3 exposure on blood urea concentration of sheep and cattle also have been observed (Phillips 2007).

In pigs, prolonged exposure to 35 and 50 ppm (24 and 35 mg/m^3) NH_3 increased weaned pigs' white blood cell count, the absolute numbers of monocytes and lymphocytes, and concentrations of serum cortisol and haptoglobin (Von Borell, *et al.* 2007). In contrast to this, no clear influence on hepatic gene expression was found in pigs chronically exposed to 20 ppm (14 mg/m^3) NH_3, despite the sentinel role that the liver plays in detecting and responding to factors affecting normal homoeostasis (Cheng, *et al.* 2014). Ammonia exposure resulted in lower concentrations of salivary cortisol and larger adrenal cortices, which may indicate down-regulated hypothalamic–pituitary axes (O'Connor, *et al.* 2010).

1.1.3.3 Behavioural responses

In simulated ship journeys, there was behavioural evidences of mucosal inflammatory responses to NH_3 in sheep and cattle, in the form of sneezing, lacrimation, and coughing (Phillips, *et al.* 2010, 2012a). Sheep exposed to NH_3 were also less active with less locomotion, pawing and panting at 45 ppm (31 mg/m^3) NH_3. In an on-ship comparison, sheep in pens with high NH_3 spent less time feeding and ruminating and held their head higher than those in pens with low NH_3, probably to avoid the higher NH_3 concentrations at lower heights (Pines & Phillips 2013).

As a non-invasive method, behavioural study integrates sensory, perceptual and cognitive processes and complement physiological investigations in research. Animals' perceptions of different environments can be investigated by preference test to record the responses from the animals' point of view (Dawkins 2003), with the assumption that animals are motivated to approach attractive stimuli and avoid disturbing stimuli.

In a test of avoidance, sheep exhibited a moderate aversion to 45 ppm (31 mg/m^3) NH_3, in comparison with fresh air, with no evidence of sensitivity being affected by previous NH_3

exposures. Although only partial avoidance was observed, the proportion of sheep preferring fresh air to NH_3 was more than 5%-20%, which has been suggested as the maximum proportion of humans that should be allowed to perceive unpleasant sensory stimulation without regulatory control (Paustenbach & Gaffney 2006; Phillips, *et al.* 2012b). Although similar research is limited in cattle and sheep, several studies have been conducted in pigs and poultry under intensive housing conditions. Laying hens showed a significant aversion to NH_3 in a free choice test demonstrated by spending more time and preferring to forage, rest and preen in fresh air rather than in NH_3 treatment (25 or 45 ppm; 17 or 31 mg/m^3) (Kristensen, *et al.* 2000). In broiler chickens, the occupancy and duration of visits to an area declined with increasing NH_3 concentrations (10 to 40 ppm; 7 to 28 mg/m^3), when tested from 30 to 46 days of age (Jones 2002). Wathes, *et al.* (2002) also observed similar avoidance, although a delay may indicate the development of a sense of malaise. The NH_3 concentration avoided by broiler fowl was suggested to be above 10 ppm (7 mg/m^3), as the NH_3 concentrations commonly found in poultry units were avoided regardless of previous experience (Jones, *et al.* 2005).

In pigs, 100 ppm (70 mg/m^3) NH_3 exposure could result in an instant but weak aversion (Jones, *et al.* 1998). While in a choice test, pigs chose to rest, sit, feed and forage more in unpolluted compartments, with aversion growing with NH_3 concentrations up to 40 ppm (28 mg/m^3), in which pigs spent just 5% of time, compared with 53% of time in 0 ppm (0 mg/m^3) compartments (Jones, *et al.* 1996). This was not an instant aversion, appearing to derive from a sense of malaise in a polluted atmosphere. Pigs exposed to 20 ppm (14 mg/m^3) NH_3 showed less play behaviour (O'Connor, *et al.* 2010), and NH_3 may also undermine their social stability, particularly when in a coincident low lighting level (Parker, *et al.* 2010).

1.1.3.4 Olfactory perception and acclimatization to ammonia

Chemical stimulation of the trigeminal nerve often combines with stimulation of the olfactory nerve to produce sensations as an overall perception of a chemical (Dalton & Jaén 2010). Although acute exposure to 40 ppm (28 mg/m^3) NH_3 had no effect on pigs' ability to detect buried odorized food, olfactory perception in 50% of pigs was interfered with by chronic exposure to 40 ppm (28 mg/m^3) NH_3, and this loss of acuity was not necessarily reversible (Jones, *et al.* 2000, 2001). Chronic exposure to 36 ppm (26 mg/m^3) NH_3 seemingly affected pigs' social discrimination, although no influence on their ability to employ olfactory cues in social recognition. Compared to the longer time pigs spent with an unfamiliar pig in fresh air, those exposed to NH_3 showed a preference for a familiar one (Kristensen, *et al.* 2001). Because of the paucity of data for livestock export, future research is required to investigate the effects of NH_3 on olfactory perception.

Limited research about the acclimatization to NH_3 has been documented, and the results are inconsistent. In humans, the inurement was developed after repeated NH_3 exposure (Ferguson, *et al.* 1977), and the change was found in odor sensitivity caused by chronic exposure to 9 ppm (6 mg/m^3) NH_3 (Holness, *et al.* 1989). Cows appeared to adapt to higher NH_3 exposure despite an increasing incidence of nasal irritation (Kertz, *et al.* 1977). Similarly, initial aversion to 100 ppm (70 mg/m^3) NH_3 in pigs was not complete or sustained, as evidenced by rapid and consistent tolerance after repeated exposure (Jones, *et al.* 1998). In contrast, there was no evidence of habituation or sensitization in sheep exposed to 45 ppm (31 mg/m^3) NH_3 in simulated ship journeys (Phillips, *et al.* 2012b). Broiler fowl showed an aversion to NH_3 concentrations commonly found in poultry units regardless of previous experience (Jones, *et al.* 2005). Because of the limited data for live export, more research is needed to investigate whether desensitization occurs in sheep and cattle, as it could be argued that animals would acclimatize over the duration of the voyage, with a consequent reduction in discomfort.

1.1.4 Threshold levels of atmospheric ammonia for humans

Short-term exposure may allow higher threshold values, but little is known about the long-term influence of NH_3 in the working environment. However, lower concentrations are always preferable to higher concentrations, both for humans and livestock. The odor threshold value of NH_3 found in confinement houses is 4.7 ppm (3 mg/m^3), while the lowest toxic value is 25 ppm (17 mg/m^3) (Tamminga 1992). To protect workers against irritation to the eyes and the respiratory tract and minimize discomfort, 25 ppm (17 mg/m^3) and 35 ppm (24 mg/m^3) are recommended as the TWA exposure level and short-term exposure limit (15 minutes) of NH_3 respectively, by the American Conference of Governmental Industrial Hygienists (ACGIH 1994), the National Institute for Occupational Safety and Health (NIOSH 2005), and NOHSC (1995). In comparison, the Occupational Safety and Health Administration (OSHA 2012) sets 50 ppm (35 mg/m^3) as the PEL of NH_3 for humans. While the American Industrial Hygiene Association (AIHA 2014) Emergency Response Planning Guidelines (ERPG) suggest 25 ppm (17 mg/m^3) as the maximum NH_3 exposure level, below which most individuals can be exposed for at least 1 hour without suffering more than mild, transient health effects (ERPG-1). 100 and 50 ppm (70 and 35 mg/m^3) are suggested as the maximum NH_3 levels that most individuals could be exposed for up to 1 hour without experiencing or developing irreversible or other serious health effects or symptoms that could impair their ability to take protective action (ERPG-2). For exposure up to 1500 ppm (1045 mg/m^3) (ERPG-3), although most individuals could be exposed for up to 1 hour without experiencing or developing

life-threatening health effects, it is inferred that their ability to take protective action would be impaired.

1.1.5 Threshold levels of atmospheric ammonia for animals

For live export, there is no universally applied maximum NH_3 concentration, except for some recommendations from different studies. Tudor, *et al.* (2003) recommended that NH_3 levels should be kept below 20 ppm (14 mg/m^3), based on preliminary lung studies in cattle exposed to 10-18 ppm (7-13 mg/m^3) or 9-16 ppm (6-11 mg/m^3) NH_3 for 9 days under simulated conditions. Costa, *et al.* (2003) recommended 25 ppm (17 mg/m^3) as an appropriate maximum NH_3 concentration for livestock shipments. However, initial clinical signs of inflammation were also found in BAL samples of cattle exposed to 22 ppm (15 mg/m^3) NH_3. In a similar study, Phillips, *et al.* (2010) suggested a critical concentration of 30 ppm (21 mg/m^3) NH_3 for steers, because nasal discharge and excessive lacrimation was seen in an acceptable number of steers (10%-20%) using the proposed criteria for humans, whereas it was unacceptable at 45 ppm (31 mg/m^3) (35%-40%). Increased coughing and significant inflammatory cell levels in bronchoalveolar fluid were found at 30 ppm (21 mg/m^3), as well as increased pulmonary macrophage activity at 15, 30 and 45 ppm (10, 21 and 31 mg/m^3). The same researchers concluded that, although there was currently limited evidence on which to base a critical concentration for sheep, it should be the same as that proposed for steers (30 ppm, 21 mg/m^3) until further information is available (Phillips, *et al.* 2012a).

In poultry houses, the United Egg Producers (UEP) guidelines recommended aerial NH_3 concentration should ideally be less than 10 ppm (7 mg/m^3) and should not exceed 25 ppm (17 mg/m^3), and temporary excesses should not adversely affect birds' health (UEP 2014). Consistent with this recommendation, Jones, *et al.* (2005) suggested NH_3 concentrations in a broiler house should not exceed 10 ppm (7 mg/m^3) commonly found in poultry units, and a threshold value of 25 ppm (17 mg/m^3) for aversion to NH_3 was suggested by Kristensen, *et al.* (2000) based on the behavioural changes in laying hens. In pigs, although Urbain, *et al.* (1994) proposed 15 ppm (10 mg/m^3) as the maximum NH_3 concentration to be tolerated in pig buildings, there has been little effort to lower the Commission Internationale de Genie Rural (1984) recommendation of 20 ppm (14 mg/m^3) for pigs.

Limited research on the establishment of acceptable limits of NH_3 concentrations for cattle and sheep during live export, and inconsistent existing results, may be the reasons for the higher thresholds for these species (25 ppm, 17 mg/m^3) than those proposed for pigs and poultry (10-15 ppm; 7-10 mg/m^3). In the light of the equivocal nature of the above evidence, further research is

recommended to assess fully the impacts of NH_3 exposure on the livestock welfare during live export by sea in order to determine the critical NH_3 concentration.

1.1.6 Conclusions

Ammonia exposure has major adverse consequences for farm animals, both those in sheds with restricted ventilation and high stocking densities, and those in transport vessels, where concentrations can build up to high levels, albeit usually for a shorter duration than in livestock buildings. These consequences include irritation of the mucosal membranes of eyes, nose, mouth and throat, respiratory and ocular infections and the development of a feeling of malaise that may reduce feed intake and activity. Further research is required aiming to the establishment and enforcement of exposure limits, particularly for livestock on ships during export from Australia to the Middle East, for which the evidence is less clear than for pigs and poultry.

1.2 Effects of climatic factors on livestock during export

So far, little is known about the influence of climatic factors on livestock during sea export from Australia to the Middle East. Heat stress was identified as a major contributor to poor animal welfare during live export voyages from the southern to northern hemisphere (Caulfield, *et al.* 2014). For a comprehensive introduction, please refer to the recent reviews (Phillips 2016; Collins, *et al.* 2018; McCarthy 2018; Australian Veterinary Association 2018) for more information. To estimate and minimise the risk of heat stress mortality in livestock during sea export, a Heat Stress Risk Assessment model was developed for the Australian livestock export industries (Maunsell Australia 2003). For a comprehensive introduction, please refer to the review (Ferguson, *et al.* 2008) for more information. The climatic factors taken into account by the model include the predicted wet bulb temperature at the destination or en route for the specific time of year, but the role of climatic conditions at the departure ports plays in prediction of sheep mortality risk is unknown. Therefore, further investigation based on the actual mortality occurrence on board shipments and relevant climatic conditions at departure and destination ports may contribute to the model's refinement to potentially improve the accuracy.

1.3 Regulation for environmental monitoring on shipment during live export

Australian law regulating the livestock export by sea incorporates the Australian Standards for the Export of Livestock (ASEL, DAFF 2011). Although NH_3 accumulation and heat stress have been identified as important welfare issues during sheep export by sea from Australia to the Middle East, environmental monitoring on board shipment has not been thoroughly considered and regulated by ASEL (DAFF 2011). Ammonia concentrations vary spatially on shipments due to ventilation

efficiency, temperature and the extent of faecal accumulation on the decks (Pines & Phillips, 2011). However, NH_3 measurement on ships has not been required and regulated yet by ASEL (DAFF 2011). Even for the temperature and humidity, only one average recording for each deck each day is required by ASEL (DAFF 2011), and the location for the measurement is not prescribed and could be in the best ventilated part of each deck. Thus, the measurement may not be indicative of the maximum daytime or nighttime temperature because of the diurnal and spatial variation in temperature on ships. Developing an effective sampling strategy for NH_3, temperature, and humidity measurement on board shipments may assist with future regulation for environmental monitoring on the vessels during live export from Australia to the Middle East.

1.4 Objectives and hypothesis

In relation to the knowledge reviewed in this chapter, the objective of this research project is to improve sheep welfare during live export by sea from Australia to the Middle East through:

- Exploring the effects of ammonia exposure, with a special focus on the mechanisms underlying the adverse effect on sheep feed intake;
- Investigating the climatic influences on sheep mortality during export, with a special focus on the climatic conditions at departure and destination ports;
- Developing a sampling strategy to evaluate the micro-climatic conditions on board shipments based on the data maps obtained on two typical voyages of live sheep export.

The hypotheses are:

- Sheep exposed to ammonia conditions typical of live export from Australia to the Middle East would demonstrate effects on their feed intake, nutritional behaviour, stress levels, as well as emotional states.
- Climatic conditions at departure and destination ports and the type of voyages would contribute to the prediction of sheep mortality risks during export from Australia to the Middle East.
- A sampling strategy to measure ammonia concentration, temperature and humidity on board shipments during export from Australia to the Middle East would be developed based on an analysis of measurement uncertainty.

2 Chapter 2: Behaviour and physiology of sheep exposed to ammonia at a similar concentration to those experienced by sheep during export by sea

2.1 Abstract

Ammonia accumulates in livestock accommodation, which inflames mucosal tissue to cause coughing, sneezing and lacrimation and adversely affects feed intake. This study aimed to find out why feed intake is reduced for sheep in ammonia conditions typical of live export by measuring nutritional behaviour and stress levels. Twelve sheep were randomly allocated to Ammonia or Control treatments in a changeover design with three 2-week periods. Ammonia exposure reduced feed intake and defecation time and slowed the rates of eating hay, masticating alfalfa pellets and rumination chewing. It also lengthened the pauses between swallowing and regurgitation during rumination, increased faecal corticosterone metabolites concentration on day 6, increased respiratory rate, and reduced yawning. The increase in faecal corticosterone metabolites concentration in ammonia exposed sheep was not correlated with the reduction in feed intake. The results suggest that although sheep exposed to ammonia typical of a live export shipment are stressed, this is not the reason for reduction in feed intake. They may have an irritation in the buccal cavity which retards nutritional behaviour, and causes shallow rapid breathing to minimise irritation to the lungs.

Keywords: ammonia, behaviour, feed intake, live export, physiology, sheep

2.2 Introduction

As the largest live exporter, Australia exported 1.74 million live sheep in 2017, mainly to the Middle East (DAFF 2018). Research on the causes of sheep death in sea voyages is limited. In the early years of live export 43% were attributed to inanition (Richards, *et al.* 1989). As a key indicator of animal welfare on ships (Pines, *et al.* 2007), ammonia (NH_3) is believed to contribute to this disorder because its accumulation in sheep pens decreases feed intake and body weight gain (Phillips, *et al.* 2012a). However, little is known about the mechanisms underlying these effects.

Farm livestock demonstrate avoidance behaviour in response to NH_3 exposure, which is believed to be due to the pungent and suffocating odour (Jones, *et al.* 1996; Wathes, *et al.* 2002; Phillips, *et al.* 2012b). The odour could arouse an emotional response through an impact on cognition and emotion (Kadohisa 2013), which may be measurable by animals' responses to the ambiguous cues in the cognitive bias (CB) test (Paul, *et al.* 2005; Mendl, *et al.* 2009). However, little research has investigated the impact of NH_3 exposure on the affective states of farm livestock, particularly sheep.

Another reason for reduced feed intake could be an impact on digestive efficiency. Chewing activities during eating and rumination play a crucial role in the ingestion and digestion of feed by contributing approximately 80% of the reduction in the feed particle size in ruminants (McLeod & Minson 1988; Beauchemin 1991). As a water-soluble irritant gas, mucosal irritation following NH_3 exposure has been extensively documented in pigs and poultry (Jones & Webster 1998; Kristensen & Wathes 2000), as well as in exported livestock (Phillips, *et al.* 2010, 2012a; Pines & Phillips 2013). Nasal discharge in exported livestock (Phillips, *et al.* 2010, 2012a) may lead to open mouth breathing to provide a wide airway with minimal resistance to air flow, which mimics a response to allergies, such as in humans (McLean, *et al.* 1979; Badhwar & Druce 1992). This may increase NH_3 dissolved into saliva, which is secreted in large volumes during the chewing process in ruminants (Bailey 1961). This may cause mucosal irritation in the buccal cavity, especially the mucosal surfaces covering the jaw bones (Larato 1975), which would affect chewing efficiency. In addition, flavour is perceived mainly by integrating smell and taste senses coming from the nose and mouth during the eating process (Boesveldt 2017), so NH_3 could mask the taste of food (Arnold, *et al.* 1980), reducing its palatability. However, little is known about the effects of NH_3 exposure on nutritional behaviour in sheep.

Exposure to aversive situations not only causes alterations in animals' behaviour, but also activates the hypothalamic–pituitary–adrenal axis to increase metabolic rate and energy utilisation, especially when the environmental stimuli challenges the body energy balance, such as in reduced food intake (reviewed in Mormède, *et al.* 2007). Thus, this study was aimed to use a multidisciplinary approach to confirm effects of NH_3 exposure on sheep feed intake and identify whether NH_3 affects nutritional behaviour, stress levels and emotional state of sheep.

2.3 Materials and methods

The study was conducted in the Queensland Animal Science Precinct, The University of Queensland, Australia (27.3˚S, 152.2˚E). Approval for this research was obtained from the University's Production and Companion Animals Ethics Committee (CAWE/242/14, Appendix-1).

2.3.1 Animals, housing and feed management

Habituation period

Fourteen Merino cross ewe lambs (4 months old, mean body weight (BW) 16.6 ± SD 2.4 kg) were purchased from a commercial property in Gatton, Queensland. Prior to the commencement of CB training and the formal trial, sheep were kept in a small paddock for 2 months. During this

habituation period, *ad libitum* access to water and alfalfa chaff were provided. Meanwhile, sheep were gradually introduced to pelleted sheep nuts (mean length 1.5 ± 0.5 cm and diam. 0.9 ± 0.1 cm; Riverina Stockfeeds Pty. Ltd., Gatton, Australia), which were used for daily feeding in the formal trial.

Timeline of the experimental procedures is presented in Figure 2-1. In order to obtain a valid representation of the NH_3 impact alone on sheep, this study minimized external stimuli that would adversely influence the testing procedure. In the first month, sheep were habituated to the potential stressors including the change of feed, new research facility, the researchers' presence, separation from the group, human handling and collection of faecal samples. To reduce the fear of humans, researchers walked quietly in the paddock and offered feed by hand for 30 minutes every 4 h daily for 14 days.

In the second month, CB training was conducted in a hexagonal facility, with six identical rooms (Figure 2-2). After successful habituation to the facility, two of the original fourteen sheep were excluded from the study (one because of parasite problems, the other because of a nervous disposition), leaving twelve sheep which were allocated to 6 pairs of similar BW (mean 24.5 ± SD 1.7 kg). Randomly, one of each pair was allocated to Group A and the other Group B, both going through a series of Control and NH_3 treatments. Two waiting and testing rooms were randomly allocated to each treatment group of sheep for the entirety of the study.

Each sheep waited in the start pen in the waiting room before training, and then entered into the testing room through a roller door. During the first 3 days, sheep were habituated to the facility by entering in pairs and receiving 30 g feed reward (sheep nuts) scattered on the floor in the centre of the room. During the following 4 days, sheep entered individually and received 15 g feed reward scattered on the floor and the other 15 g in a bucket placed in the centre of the room. The number of entries was increased from one to five consecutive entries by the third day.

Cognitive bias training

After the first week, sheep within group were randomly assigned either left (n = 3) or right (n = 3) corners of the facility for positive training, to prevent the potential influence of side biases. Positive training sessions were performed five consecutive times daily for each sheep, by allowing the sheep to approach the feed bucket placed in the allocated corner and eat 15 g sheep nuts, without a time limit.

After 1-week positive training, negative training sessions were incorporated by splitting the five sessions into three positive and two negative. A pseudo-random order was used to ensure no more than two negative or positive events were consecutive. In each session, the first 4 sessions were randomly assigned to positive or negative, but the fifth was always positive to prevent a negative feeling after the final test, which might make subsequent training difficult.

Negative training allowed the sheep to approach an empty feed bucket placed in the corner opposite the positive location (P), with a time limit of 25 s. A 30 cm radius was measured out and marked around all bucket positions beforehand. Concurrent with a sheep's entry into the circle, a dog barking soundtrack (Id Number 10685628, Sounddogs 2018) was played as the only negative reinforcement in the first week of negative training through a wireless speaker (Punch Box Bluetooth Speaker, Xoopar, China) hidden in the bucket. Then, in order to strengthen the negative reinforcement, at the same time water was shot at the sheep's body from a 100 ml syringe operated by an experimenter hidden behind the panel at the negative location (N) as an additional negative reinforcer. The negative reinforcers continued until the sheep retreated. P and N were equidistant (3 m) from the start pen. The training with both negative reinforcers lasted for 5 days. Sheep were classified as trained only if they only approached the positive location (100%) and no negative locations (0%) for three consecutive blocks of training sessions (Doyle, *et al.* 2010). The twelve sheep in this study were not trained successfully until the last 5 days with both negative reinforcers.

After each entry, the sheep was returned to the start pen to wait for a 2-minute interval, while the experimenter prepared the bucket for the next session. To ensure the choice made by sheep was not due to behavioural, audible or olfactory cues, but the result of learning the paradigm, the preparation for a negative session included the experimenter pretending to prepare the bucket by filling it with 30 g feed in the same way as for a positive session and subsequently removing the feed quietly. Then the experimenter walked between two locations (P and N) 5-7 times, as for positive sessions. After each block of five training sessions, the sheep was released back to the holding room. When the six sheep in each group had all completed their training, they were released back to the paddock.

During the CB training and testing, two video cameras (model 287 K-32HCF, Kobi CCD, Ashmore, Australia) were installed on opposite sides of the testing room (Figure 2-2). The images of training and testing were recorded by a digital video recorder (Kobi H.266, Model XQ-L 900H, Ashmore, QLD, Australia). A steel panel frame covered with black PVC was fixed behind the five bucket locations. One experimenter was responsible for shooting the water, and in the testing room,

the other experimenter operated the roller door, put the sheep in the start pen, prepared the buckets, and controlled the dog barking sound with cell phone. All sheep had been habituated to being handled by the experimenters in the waiting and holding rooms prior to the start of the CB testing.

Animal management in the trial period

The experiment was a changeover design conducted for 3 periods of 14 days in two climate-controlled rooms (each 9.5 m long, 4.8 m wide, 3 m high). Sheep were individually exposed to the NH_3 and Control treatments in the following sequences, Group A: NH_3 treatment, Control, and NH_3 treatment; Group B: Control, NH_3 treatment, and Control. Each group of sheep were randomly allocated to six individual pens (2.7 m × 1.3 m) in one room. Two feed bins and a water bucket were provided in each pen, occupying 0.24 m^2 of the available pen space. Each room was lit on a 12:12 L:D schedule, but with a minimum lighting in the dark (10% lighting), for safety.

Each sheep was offered 400 g of the same concentrate pellets as used in the habituation period and *ad libitum* access to a 50:50 mixed hay of Rhodes grass (*Chloris gayana*) and alfalfa (*Medicago sativa*), which was predicted to meet the energy requirements for the maintenance and BW gain of 250 g/day (Aldermann, *et al.* 1975). Daily hay intake was evaluated by weighing the hay provided and refusals of each sheep. Hay refusals were weighed at 08:30 h prior to room cleaning, and 10% more than the hay intake of the previous day was given to each sheep. Daily hay dry matter intake (DMI) was calculated from the daily hay intake and the DM content of hay samples. The DMI in the last 2 days of each period was not included for analysis due to the feed deprivation before the CB test. Representative samples of offered feed and refusals were collected on days 1, 8, 12 and pooled periodically and frozen at - 20 °C until chemical analysis. Samples of all feeds utilized in the experiment were analyzed for nutrient concentrations (Table 2-1, Feed Central Pty. Ltd, Toowoomba, Australia). Access to water was *ad libitum* and daily intake estimated by weighing the water in the bucket before and after daily refilling.

2.3.2 Micro-climatic measurements

A physiologically-validated maximum NH_3 concentration of 30 ppm (21 mg/m^3) proposed for live steer export (Phillips, *et al.* 2010) was used as the target NH_3 concentration in this study, with supporting evidence from previous NH_3 research on sheep export (Phillips, *et al.* 2012a). To achieve 30 ppm (21 mg/m^3) of NH_3 treatment, 350 ml of domestic cloudy ammonia (20 g NH_3/L, Coles Home Brand, Australia) was evenly sprayed on the walkways beside the pens with a 100 ml syringe every morning after room cleaning. In addition, at two locations in the walkway, cloudy ammonia stored in a drip bottle and dripped through an infusion set at 1 drop/second into a bucket

(0.30 m diam., 0.38 m high). The volume of domestic cloudy ammonia and initial dripped rate required to achieve the target NH_3 concentration was determined prior to the trial. Two fans were used near the buckets to enable the NH_3 released to be evenly distributed in the room, but avoiding directing wind onto the sheep. A gas detector (GasAlertMicro 5 IR, Thermo Fisher Scientific, Brisbane, Australia) was used 4 times/day (08:00, 09:30, 12:00 and 17:00 h) to measure NH_3 concentration at sheep height. Three sites located in the middle of three walkways along side of sheep pens were selected for NH_3 measurement. The gas detector was calibrated prior to the trial and fresh air calibrations were conducted before each measurement. The dripping rate of cloudy ammonia was adjusted and extra cloudy ammonia was sprayed whenever necessary according to the NH_3 level monitored in the sheep pens. The effectiveness of these strategies to maintain NH_3 concentration in the climatic room was tested by measuring NH_3 every hour during daytime in 3 days prior to the trial. Ammonia in the Control room was controlled below 4 mg/m^3, and ventilated to the atmosphere as necessary.

In each climatic room, two data-logging devices (HOBO UX100-011 Temp/RH 2.5%; Onset Computer Corporation; Bourne, MA, USA) were used for four daily recordings of temperature (T) and relative humidity (RH) each day at sheep height. The ventilation system inside the rooms was operated only at 08:30 h - 09:30, 23:00 - 23:15, and 04:00 - 04:15 h, to control T and RH and also prevent excessive NH_3 liberation to the atmosphere. Wind speed was measured as 0.04 m/second in each pen in the NH_3 and Control treatments.

2.3.3 Behaviour recording

Sheep behaviour was video recorded continuously for a 48-hour period on days 7 and 8 in each period. Same video cameras as used in the CB training were set to cover pairs of adjacent pens. On the same days, ruminating behaviour was manually observed as the chewing rate and duration of the pause between swallowing and regurgitation for 10 periods of 60 seconds between 14:00 and 19:00 h by an experimenter, who entered the room and sat quietly 10 minutes before recording to allow sheep to adjust to the human presence. Cowlog 3.0.2 software (Hänninen & Pastell 2009) was used to code behaviours (Table 2-2). Hay eating rate and eating rates in different head positions were calculated as daily DMI for each sheep/relevant eating duration. Rumination chewing rate was calculated as ruminating chews/rumination time.

2.3.4 Feed palatability test

A 6-minute feed palatability test (Rapisarda, *et al.* 2012) was conducted on days 10 and 11 of each trial period. Starting at 09:30 h, simultaneously in the two treatment rooms sheep were tested

successively in each pen, with behaviour recorded by same video cameras as used in the CB training. Two feeds were separately offered, one of high quality (400 g alfalfa pellets, mean length 1.1 ± 0.2 cm and diam. 0.5 ± 0.04 cm; Lockyer Alfalfa Products Pty. Ltd., Gatton, Australia) and one of low quality (200 g sorghum chaff, chopped into 1-3 cm lengths). On the two test days no daily allowance of concentrate feed was provided. On day 1 alfalfa pellets were tested first and sorghum chaff second, on day 2 the reverse order was used. Representative samples of offered feed were collected and pooled periodically and frozen at - 20 °C until chemical analysis. Eating duration, masticating and prehension rates were recorded for alfalfa pellets. For sorghum chaff, only eating duration and masticating rate were recorded due to a lack of clear video footage. Cowlog 3.0.2 software was used to code the eating behaviours. Three 10-second video clips, when the sheep was eating with its head down, were sampled every 2 minutes to calculate the number of manipulative jaw movements (prehension bites) and a separate set of video clips when the sheep was chewing with its head raised was used to calculate the number of chewing jaw movements (mastication bites).

2.3.5 Physiological measurements

Faecal sampling and processing

Owing to the fact that stress plays a key role in the exhibition of sheep behaviour, glucocorticoid measurement was conducted in the first 6 days to minimize the potential influence of external stimuli on sheep behaviour, including sheep handling and manual faeces collection. Taking into account the lag time of faecal glucocorticoid metabolites excretion, the measurements on days 3 and 6 were considered to reflect sheep responses to more immediate and longer term stress, respectively.

On days 3 and 6 in each period, faecal samples were collected manually per rectum into a zip-lock plastic bag between 08:30 and 09:30 h, for the detection of cortisol and corticosterone metabolite concentrations. Faecal samples collected were immediately stored in an ice box, and then frozen at -20℃ until analysis. Prior to the extraction, faecal samples were thawed and homogenized by thorough mixing within the bags. Then, samples were moved onto a spot plate with labels and placed in an oven at 65° C for 24 h to complete the desiccation process. A 0.5 ± 0.01 g dry subsample was weighed into a glass scintillation vial and 5 ml of 80% methanol added. The mixture was vortexed until homogenised, and left overnight on a rotating shaker, prior to centrifugation at 1000*g* for 10 minutes at 18 ℃. The supernatant was decanted into labelled extract storage vials and stored frozen (- 20 °C) prior to analysis.

Faecal cortisol and corticosterone metabolites measurement

Faecal cortisol and corticosterone metabolites (FCM and FCSM, respectively) concentrations were determined using enzyme immunoassay procedures (Palme & Mostl 1997; Keeley, *et al.* 2012, respectively). There were minor modifications for FCSM assay: in addition to the goat anti-rabbit globulin (Arbor Assays, USA; A009) used to pre-coat the microtire plates, the assay used corticosterone antibody (stock dilution: 1:200; dilution rate: 1:120,000; C Munro, UC Davis, CA, USA), corticosterone horseradish peroxidase conjugated label (stock dilution: 1:200; dilution rate: 1:250,000; C Munro, UC Davis, CA, USA), and corticosterone standards (0.1 - 50 ng/ml; Steraloids, USA, Product #Q1550-000). Major cross-reactivities (> 5%) for the antibody were corticosterone 100% and deoxycorticosterone 14.3%. A serial dilution of pooled faecal samples demonstrated parallelism with the standard curve. The dilution rate for faecal samples (1:10) was based on the dilution ratio of pooled samples with 50% binding on the parallelism curve. Faecal samples were analysed in duplicate and the assay sensitivity was 0.1 ng/ml. Plates were read at 450 nm (reference filter: 630 nm) using a microplate spectrophotometer reader (Epoch, Winooski, VT, USA) with Gen5 software (Biotek, USA). The intra- and inter-assay CV for corticosterone assay were 2.84 % and 2.83 %, respectively.

Respiratory rate and rectal temperature measurement

Respiratory rate (RR) were manually recorded as the time taken for 10 breaths (using a stopwatch and counting uninterrupted flank movements) at 17:00 h each day except those days for general behaviour recording. Rectal temperature (RT) was measured using a digital thermometer (S+M; Tollot Pty Ltd, Blacktown, NSW, Australia) between 08:30 and 09:30 h on days 3, 6 and 11 to test for pyrexia as NH_3 is known to cause an inflammatory response at mucosal surfaces (Phillips, *et al.* 2010, 2012a; Pines & Phillips 2013).

2.3.6 Cognitive bias test

Sheep were subjected to two CB tests, which measure affective state, on day 14 of each period and day 1 of the following period. After the CB test on the second day, NH_3 and Control treatments were switched to start the following trial period. Two pairs of adjacent rooms (waiting and testing) in the hexagon were allocated at random to Group A and Group B. Prior to testing of the Group receiving NH_3 in each period, NH_3 concentrations in the holding, waiting and testing rooms were elevated to 30 ppm (21 mg/m^3) for CB training and testing. To achieve this, 150 ml of cloudy ammonia was initially evenly sprayed on the floor with a 100 ml syringe. Urine and faeces in the holding room enabled the NH_3 level to be held at 30 ppm (21 mg/m^3) without further applications, but in the waiting and testing rooms extra dosage (20 ml) were added as necessary following

measurement every 15 minutes at sheep height. Sheep were feed-deprived before CB test. Any feed rewards given during test procedures were included in their daily rations.

Prior to the tests in each period, sheep were re-trained 3 times to ensure the task was remembered. On each testing day, approach latency responses of the sheep were tested with the bucket located at two learnt locations (P and N) and three ambiguous locations between them: 0.5 m (an ambiguous, partially positive location, AP), 1.0 m (a middle ambiguous location, M) and 1.5 m (an ambiguous, partially negative location, AN) far from the positive location, respectively. Five locations were presented in a randomly generated order, which was consistently used for all the sheep tested that day. Initially, the buckets were presented at first the positive and second the negative locations to remind the sheep of the task learnt previously. Then three ambiguous locations were presented in the order: AP→AN→M on day 14 and M→AP→AN on day 1 of the following period. A time limit of 25 seconds was set for sheep to respond to each location, and their response was video-recorded. After each test sheep was returned to the start pen. When an entire group of sheep had finished their tests, they were returned to the treatment room. The order of sheep groups during the 2 days of tests in each period was counterbalanced to avoid feed deprivation length bias.

The mean approach latency in seconds to approach each location (P, AP, M, AN, N) in each period was measured for each sheep, and responses to the AP and M test locations analysed, since no sheep approached the AN bucket location. The difference in approach latency to the ambiguous locations could be due to the intrinsic differences between sheep, so we adjusted each sheep's latency to each ambiguous location by taking into account its mean 'baseline' latency to reach the P and N locations following the equation of Mendl, *et al.* (2010):

$$\text{Adjusted score} = \frac{\text{(mean latency to test location – mean latency to P location)}}{\text{(mean latency to N location – mean latency to P location)}} \times 100$$

2.3.7 Statistical analysis

Statistical analysis was carried out in Minitab software (Version 17.0; Minitab Inc, State College PA, USA). General linear models of the data were created for analysis of variance. For each measurement, the mean value of each period was calculated for each sheep. The linear components of the model contained fixed term (treatment) and random factors (period and sheep). A normal distribution of residuals was verified with the Anderson-Darling test. Optimal Box-Cox transformations were necessary as a non-normality adjustment of the initial data for hay eating rate (lambda (λ) = −1), head-down eating rate (λ = −1), head-level eating rate (λ = 0), pawing (λ = 0),

adjusted latency score to approach location AP ($\lambda = 0$), and FCM concentration on d 3 ($\lambda = -0.5$). We added 1 to each data value before Optimal Box-Cox transformation for body ($\lambda = 0.25$) and head rubbing ($\lambda = 0.21$). Least squares means were used for comparison. Results were considered significant at 5% probability level. Pearson correlations were used to compare physiological and cognitive bias variables by the Anderson-Darling test as long as the data was normally distributed, otherwise a Spearman rank correlation was used.

2.4 Results

2.4.1 Micro-climatic conditions measurements

When the NH_3 treatment was applied, mean NH_3 concentration was 20.88 mg/m^3, close to the target of 30 ppm (21 mg/m^3), with the Control treatment was close to zero (1 mg/m^3) (Table 2-3). Mean T and RH were slightly higher in the NH_3 treatment than the Control treatment.

2.4.2 Feed and water consumption, and general behaviour

Ammonia exposure decreased daily hay DMI ($P = 0.005$) from day 4 until day 12 (Table 2-4, Figure 2-3), with an increased total eating time ($P = 0.02$) and decreased hay eating rate ($P = 0.01$). Specifically speaking, NH_3 treatment decreased eating rate with the head down ($P = 0.01$) with more time spent absolutely or proportionately ($P = 0.01$ and 0.03, respectively). No differences were found in eating rate or time with the head level or up (P = rate, level 0.67, time, level 0.64, rate, up 0.47, and time, up 0.93, respectively), but sheep spent proportionately less time with their heads level or up ($P = 0.04$ and 0.03, respectively). Moreover, sheep exposed to NH_3 reduced their eating time while walking, as a % of total eating time ($P = 0.04$), with a trend of increased proportion of eating time while standing ($P = 0.06$). Ammonia exposure had no influence on water intake ($P = 0.71$) or total drinking time ($P = 0.93$).

Besides feeding, NH_3 treatment decreased both the number of ruminating chews ($P = 0.01$) and rumination chewing rate ($P = 0.01$), but increased the pause between boluses, compared to those in the Control treatment ($P = 0.002$). Sheep exposed to NH_3 also demonstrated a trend towards a decreased time spent ruminating ($P = 0.06$).

Ammonia treatment increased the time sheep spent standing ($P = 0.03$), but had no effect on total walking or lying time ($P = 0.72$ and 0.21, respectively). In addition, NH_3 treatment resulted in a higher proportion of idling that was spent lying ($P = 0.01$). Ammonia exposure reduced daily defecating time ($P = 0.05$), but had no effect on urinating time ($P = 0.40$). No treatment effects were found on time spent scratching ($P = 0.79$) or body rubbing ($P = 0.23$), or pawing frequency ($P =$

0.75), however, head rubbing was reduced in the NH_3 treatment ($P = 0.05$). Sheep exposed to NH_3 had approximately half the frequency of yawning compared to those in the Control treatment ($P = 0.02$).

2.4.3 Feed palatability test

Ammonia treatment decreased masticating rate of alfalfa pellets ($P = 0.00$), with no effect on the prehension biting rate and eating duration of alfalfa pellets ($P = 0.87$ and 0.73, respectively) (Table 2-5). Ammonia treatment had no effect on the masticating rate or eating duration of sorghum chaff ($P = 0.47$ and 0.68, respectively).

2.4.4 Physiological measurements

There was no effect of NH_3 on FCM levels on either day 3 or 6 ($P = 0.21$; $P = 0.64$), or FCSM level on day 3 ($P = 0.13$) but on day 6 FCSM levels were higher in sheep exposed to NH_3 ($P = 0.04$) (Table 2-6). In NH_3 exposed sheep there was no correlation between FCSM on day 6 and DMI (Pearson correlation coefficient (CC) = 0.005, $P = 0.99$), but in the Control treatment there was (Pearson CC = -0.54, P = 0.03). Respiratory rate of sheep exposed to NH_3 was increased compared to the Control group ($P = 0.00$), but RT was not affected by NH_3 treatment ($P = 0.25$). Within the NH_3 treatment, RR was positively correlated with FCM level on day 3 (Spearman Rank CC = 0.52, $P = 0.03$), but not in the Control treatment (Spearman Rank CC = 0.23, $P = 0.36$).

2.4.5 Cognitive bias test

Adjustment for the latency to approach the ambiguous locations did not affect the significance of results compared with analysis based on the unadjusted form. Ammonia treatment had no effect on the cognitive response to either AP ($P = 0.81$) or M ($P = 0.46$) ambiguous locations in this study compared to those in Control group (Table 2-7). Within the NH_3 treatment, sheep with high FCSM on day 6 were faster in making the decision to approach the M ambiguous location (Spearman CC = -0.60, $P = 0.01$), but not in the Control treatment (Spearman CC = 0.37, $P = 0.16$).

2.5 Discussion

Ammonia concentration in the NH_3 treatment was close to the target of 30 ppm (21 mg/m^3). The lower temperature and RH in the Control treatment was probably related to the additional ventilation to control the NH_3 level below 4 mg/m^3. Although the experiment was a changeover design conducted for 3 periods, earlier investigation of the avoidance of ammonia by sheep

demonstrated that there are no carryover effects for prior ammonia exposure of sheep, at about 30 ppm (Phillips, *et al.* 2012b).

2.5.1 Ammonia exposure and chewing efficiency during eating

Reduction of daily hay DMI and no effect on water intake in sheep exposed to NH_3 is consistent with previous research (Phillips, *et al.* 2012a). The decreased hay DMI was associated with an increased eating time and therefore the hay eating rate decreased by about one third. This could be due to reduced palatability, but palatability does not usually cause intake modification if no choice among feeds is offered to the sheep (Mertens 1994), and only hay was fed *ad libitum* in this study. Therefore, reduced chewing efficiency during eating could be the reason for reduced intake. Jaw movements during eating include gathering hay into the mouth without chewing (prehension), chewing the hay (mastication), and combined gathering and chewing (prehension/mastication) (Penning, *et al.* 1984). Sheep manipulating new bites or with existing feed in their mouths mainly have bouts of a few chews (short sequences) with their heads down, but also bouts of 5+ chews (long sequences) with their heads lifted (Laca, *et al.* 1994). The increased proportion of time NH_3 sheep spent eating with their heads down and therefore the decreased eating rate may reflect an increase in the number of short chewing bouts. Conversely, the reduced proportion of time NH_3 sheep spent eating with their heads level or up may have decreased the number of long chewing bouts. Any irritation in the buccal cavity resulted from NH_3 exposure may have mitigated against long bouts of chewing if this caused pain. Further support for this theory comes from the results of the feed palatability test. The reduced mastication rate of alfalfa pellets for sheep in the NH_3 treatment is likely to have been of long chewing bouts, since the videos for this measurement were recorded when sheep were chewing with their heads raised. Although the decreased hay eating rate with the head down could be due to a reduced prehension biting rate, this was unlikely. First, NH_3 had no effect on prehension biting rate of alfalfa pellets, although this was not measured for hay. Second, prehension is more important for grazing sheep, and the hay used in this study was already harvested and chaffed. Third, sheep perform gathering hay motions only with their teeth, lips and head, without using the tongue as a prehension organ (Laca, *et al.* 1992; Woodward 1998). This means prehension activity might be less affected by mucosal irritation in the buccal cavity with NH_3 exposure. Alternatively, the head position difference between the NH_3 and Control treatments may be also because NH_3 is lighter than air, and therefore usually increases with height above the floor (Brannigan & McQuitty 1971). However, when faeces are not removed from the floor, such as on a ship, NH_3 concentration decreases with height above the floor (Pines & Phillips 2011), and sheep have been recorded to elevate their heads on a ship (Pines & Phillips 2013).

In the feed palatability test, NH_3 treatment had no effect on mastication rate of sorghum chaff, probably because of its texture. According to Gisel (1991), chewing time required for a particulate food before it can be swallowed is determined by its texture, which is quantitatively related to the chewing activity in ruminants (Sudweeks, *et al.* 1981). Compared with cereals, proteinaceous feedstuffs require greater effort to perform the chewing process due to their hygroscopic property (Solà-Oriol, *et al.* 2009). Sorghum chaff may require less saliva to form feed boli before they can be swallowed, compared to alfalfa pellets, which has higher protein content (Table 2-1). The limited 6 minutes of the test and feeding motivation of sheep before daily feeding in the morning (Forbes 2010) might explain why NH_3 treatment had no effect on the eating duration of alfalfa pellets or sorghum chaff.

2.5.2 Ammonia exposure and chewing efficiency during rumination

After the initial chewing during eating, rumination chewing plays a major role in reducing the feed particles size (> 1 mm) in the rumen to < 1 mm, which efficiency is determined by the number of ruminating chews (jaw movements) per minute (Dellow & Barry 1991). Ammonia treatment decreased the efficiency of ruminating chewing indicated by a reduced daily ruminating chews and ruminating chewing rate, as well as a trend towards a decreased ruminating time. The declined hay DMI in NH_3 sheep may also be due to the decreased efficiency of ruminating chewing, since these two factors are intrinsically linked (Perazzo, *et al.* 2016). The critical threshold size for particles to passage through the reticulo-omasal orifice into the omasum of sheep is 1 mm (Reid, *et al.* 1979; Poppi, *et al.* 1980; Domingue 1989). Therefore, decreased rumination efficiency in the NH_3 treatment might indicate an increased proportion of particles > 1 mm in the rumen. This could adversely affect the clearance of ruminal digesta, due to the filling effect of neutral detergent fibre and reduced exit of small particles from the rumen (Ulyatt, *et al.* 1986; Berchielli, *et al.* 2011). Only a small percentage of particles > 1 mm leave the rumen and reach the faeces, < 1.5 % total faecal DM (Domingue 1989), which, together with the reduction in hay DMI may explain the reduced daily defecating time in NH_3 exposed sheep.

2.5.3 Discomfort related to ammonia exposure

A sense of discomfort might be developed in NH_3 sheep when moving the feed around in the buccal cavity during chewing, due to the mucosal irritation caused by NH_3 dissolved in saliva. Ammonia exposure prolonged the time interval between swallowing each bolus and regurgitating the next, indicating possible irritation of the throat (Cometto-Muniz & Cain 1992), oral pharynx, oesophagus, and stomach, as in humans after imbibing liquid ammonia (Sugawa, *et al.* 1981). Yawning, as an indicator of comfort and relaxation, is a typical self-care behaviour observed frequently during rest

periods (Wemelsfelder & Farish 2004; Abdul Mateen, *et al.* 2017). The decreased daily yawning frequency in NH_3 exposed sheep might be attributed to discomfort evoked by mouth opening and extreme jaw movements (Sarlani, *et al.* 2005). Similarly, decreased head rubbing time by the NH_3 treatment may be due to the discomfort associated with rubbing the cheeks against the pen furniture. Increased proportion of idling spent lying down and less eating while walking perhaps indicate a general feeling of 'malaise' caused by the NH_3 treatment, as reported in poultry and pigs (Jones, *et al.* 1996, 1998, 2005).

Reduced hay DMI in NH_3 exposed sheep was probably not attributable to the impairment of smell (Jones, *et al.* 2000) and taste, since feed intake in sheep with both taste and smell removed is similar to that of normal sheep (Arnold, *et al.* 1980). However, in humans at least, the taste is inversely related to eating rate (Boesveldt & de Graaf 2017), and irritation in the mouth could also cause taste dysfunction (Mann 2002). Thus, further investigation on the influence of NH_3 exposure in the taste of sheep is needed.

2.5.4 Ammonia exposure and physiological responses

Contrary to the dominant glucocorticoid/interchangeability assumption, a lack of correlation between corticosterone and cortisol has been recently reported in some mammalian species, which were traditionally classed as cortisol-dominant (Hancock 2010; Koren, *et al.* 2012). Taking into account the lag time of FCSM excretion, the elevated FCSM on day 6 in NH_3 exposed sheep may be because corticosterone is a better index of chronic, longer term stress compared to cortisol (Koren, *et al.* 2012; McCorkell, *et al.* 2013; Gong, *et al.* 2015). A longer incubation in the NH_3 sheep's body might be the other reason for increased FCSM on day 6, because of the shortened daily defecating duration, which might indicate a reduced faecal output (Möstl, *et al.* 1999). No difference in FCSM on day 3 may be because of the lag time between the increase of blood glucocorticoids levels and the rise of the faecal metabolites concentrations. This is determined by the passage rate of digesta between the bile duct and the rectum that was conceivably reduced because of low chewing efficiency (Palme, *et al.* 1996; Wasser, *et al.* 2000). Although the elevated FCSM on day 6 in NH_3 exposed sheep might be expected as a physiological response to the DMI reduction, no correlation between these two variables was found. This suggests that the reduction in feed intake was not due to stress or any lipogenic effects of the FCSM. In the Control treatment, there was a negative correlation between DMI and FCSM, at least on day 6, a correlation which has also been reported in cattle previously during a 72-h fast (Mills & Jenny 1979). In the cattle study, it was thought to derive from the ketogenic action of glucocorticoids, allowing release of free fatty acids from adipose tissue.

In contrast to corticosterone, cortisol is used as a better index of acute stress (Vera, *et al.* 2011; McCorkell, *et al.* 2013; Gong, *et al.* 2015), and does not reflect the overall stress response over a long period (Palme 2012). Therefore, a lack of difference in FCM levels on either day 3 or 6 might be because of a reduction in blood cortisol level either returning to, or falling below baseline values at that time (Fordham, *et al.* 1991; Ley, *et al.* 1991; Fisher, *et al.* 1997). In the light of the equivocal nature of the above evidence, further research is needed with more frequent sampling points throughout the trial phase.

Respiratory rate was increased by 28% in sheep exposed to NH_3, which may be rapid, shallow breathing to minimize irritation to the lungs (Banister, *et al.* 1949; Matsumoto 1989). The positive correlation with FCM in the NH_3 treatment on day 3 suggests that the RR increase was associated with stress in this treatment. The RT was similar between the NH_3 and Control treatments, which was close to the approximate mean daytime RT of sheep (39 ℃), and within the normal range of 37.5 to 40.5 ℃ (Terrill 1968; Esmay 1978; Mount 1979).

2.5.5 Ammonia exposure and cognitive responses

New evidence is suggesting that the CB test can differentiate between anxiety disorders and depression, based on animals' responses to different ambiguous locations. Compared to the anxiety-like state displayed by a pessimistic response to AN and M ambiguous cues, a depression-like state is in addition displayed by less optimistic response to AP ambiguous cues (Salmeto, *et al.* 2011; Hymel & Sufka 2012). In this study, the NH_3 treatment had no effect on sheep's response to either AP or M ambiguous locations, compared to the Control group. However, the faster approach of some NH_3 exposed sheep with high FCSM concentrations to the M ambiguous location may mean that they were more stressed and anxious, and previously researchers have suggested this connection with a decreased decision-making time (Burman, *et al.* 2009; Brydges, *et al.* 2012). The failure of sheep to approach the AN bucket location is probably because the two negative reinforcers during training were too strong. Further work is needed to test the effect of NH_3 exposure on cognition in sheep, particularly to investigate whether anxiety is induced. Latencies to respond should be measured and whether buccal cavity irritation might affect the responses.

2.6 Conclusions

This study demonstrated a measurable influence of 30 ppm (21 mg/m^3) of ammonia exposure on sheep feed intake and related feeding and ruminating behaviour, with a reduced intake and lower chewing rates during eating and rumination. Discomfort while chewing caused by the irritation in

the buccal cavity and/or impaired taste are speculated as the physiological mechanisms involved. Increased respiratory rate in ammonia exposed sheep and its correlation with faecal cortisol metabolites level on day 3 within Ammonia treatment indicated that in some sheep ammonia caused stress and increased rapid, shallow breathing, probably to minimize irritation to the lungs. Increased faecal corticosterone metabolites concentration on day 6 in ammonia exposed sheep and its correlation with sheep's response to the middle ambiguous location within Ammonia treatment may indicate that ammonia exposed sheep were more anxious. Further research is required to investigate the effects of ammonia exposure on buccal cavity health, taste sensations, and anxiety in sheep to understand the effects of ammonia more fully.

2.7 Tables and figures

Table 2-1. Nutrient composition of feeds used for basic ration (hay and sheep concentrate) and palatability tests (sorghum chaff and alfalfa pellets) given to sheep (n = 12) in Ammonia and Control treatments

Nutrient content, DM basis	Mixed Rhodes grass and alfalfa hay	Sheep concentrate	Sorghum chaff	Alfalfa pellets
ME, MJ/kg	9.20	12.0	8.40	9.50
CP, %	15.9	21.9	7.20	20.6
Degradable protein, % of CP	68.7		59.4	
Acid detergent insoluble CP, %	1.10		0.30	
Neutral detergent insoluble CP, %	4.60		2.50	
Non-fibre carbohydrates, %	26.6	39.7	18.8	24.8
Ethanol soluble carbohydrates, %	8.50		5.60	
Fat, %	2.60	4.30	1.50	2.50
Digestible DM, %	61.5		62.9	
NDF, %	48.3	22.8	66.2	41.9
ADF, %	33.7	10.1	41.1	36.0
TDN, %	57.8	73.0	56.1	55.8
Starch, %	2.00	29.3	1.40	0.40
Water soluble carbohydrates, %	10.4		9.00	
Lignin, %	6.20		4.60	10.3
Ash, %	11.1	12.7	8.80	10.1
DM, %	89.6	89.7	91.1	91.4
Calcium, %	0.90		0.60	1.22
Phosphorus, %	0.30		0.20	0.26
Potassium, %	2.10		1.70	1.91
Magnesium, %	0.30		0.40	0.28
Chlorine, %	0.90		1.40	
Sulphur, %	0.20		0.10	0.29
Sodium, %				0.15

Iron, ppm	532
Zinc, ppm	30.7
Copper, ppm	8.90
Molybdenum, ppm	2.72
Manganese, ppm	95.9

Table 2-2. Ethogram and behaviour definitions used for sheep (n = 12) exposed to Ammonia and Control treatments

Behavioural class	Behaviour	Definition
Feeding	Total eating	Total time prehending and chewing hay and concentrate feeds
	Eating, head down	Eating with head held below shoulder height
	Eating, head shoulder	Eating with head held at shoulder height
	Eating, head up	Eating with head above shoulder height
	Eating while standing	Eating while standing stationary
	Eating while walking	Eating with movement of body at least half body length
Drinking	Drinking	Time sheep spent with mouth in water bucket
Rumination	Ruminating	Time spent ruminating, i.e. regurgitating a bolus and chewing it, including the time interval (pause) between swallowing one bolus and regurgitating the next
	Ruminating chews	Total number of jaw movements/min, when chewing bolus in a circular motion during ruminating
	Pause between boluses	Time interval between swallowing each bolus and regurgitating the next
Body posture	Total standing	Total time spent in an upright position on all four legs without movement with or without eating and ruminating in any head position
	Total walking	Time spent moving in a forward motion movement of the body for at least a half body length, with or without eating and ruminating in any head position
	Total lying	Total time lying down in sternal or lateral recumbency with or without ruminating in any head position

	Lying, while idling	Total time lying in sternal or lateral recumbency, while not eating and ruminating
	Lie down frequency	Frequency of moving from one knee on the floor until the lying down movement was completed
Body maintenance	Defecating	Time from when sheep raised its tail and the faeces emerged, until cessation of the action
	Urinating	Time from when sheep splayed its legs and urine emerged, until cessation of the action
	Scratching	Scratching body or head with hind leg
	Total rubbing	Total time of rubbing body on fence panels or face and neck rubbing itself or rubbing head on edge of feed bin or water bucket
	Body rubbing	Time of rubbing body on fence panels
	Head rubbing	Time rubbing head on edge of feed bin or water bucket
Other behaviours	Yawning frequency	Total frequency of yawn with the mouth open, the head and neck extended, eyes rolled or closed, followed by mouth closure
	Pawing frequency	Number of times sheep used a scooping leg movement on the ground surface or edge of feed bin.

Table 2-3. Values of microclimatic conditions (ammonia (NH_3), temperature (T) and relative humidity (RH)) in chambers used for Ammonia and Control treatments

	Treatment	Mean	SD	Minimum	Median	Maximum
NH_3, mg/m^3	Control	1.02	0.90	0.00	0.70	3.48
	NH_3	20.9	1.45	17.21	20.9	23.7
T, ℃	Control	17.4	1.80	13.3	17.4	20.7
	NH_3	19.2	1.26	16.4	19.4	21.6
RH, %	Control	77.9	9.05	53.4	79.6	94.5
	NH_3	86.0	5.73	75.4	86.2	96.2

Table 2-4. Effect of ammonia (NH_3) exposure on sheep (n = 12) daily hay and water intake, and general behaviour (transformed and back-transformed means) on days 7 and 8 when exposed to Ammonia and Control treatments

Behaviour	Control	NH_3	SEM	*F* value	*P* value
Feeding on DM basis					
Hay DMI, kg/d	0.974	0.919	0.012	9.77	0.005
Hay eating rate, g DM/min^{-1}‡	0.24	0.32	0.289	8.97	0.01
g DM/min	4.17	3.13			
Total hay and pellet eating time, h/d	3.77	4.78	0.262	6.42	0.02
Eating head down, h/d	3.22	4.21	0.238	7.42	0.01
% of total eating	74.8	87.2	3.446	5.53	0.03
head level, h/d	0.23	0.24	0.013	0.23	0.64
% of total eating	10.7	5.31	1.571	5.07	0.04
head up, h/d	0.28	0.28	0.026	0.01	0.93
% of total eating	13.1	6.36	1.876	5.46	0.03
Eating rate head down, g/min^{-1}	0.20	0.28	0.016	9.67	0.01
g/min	5.00	3.57			
head level, g/min^{0}	4.29	4.25	0.057	0.19	0.67
g/min	73.0	70.1			
head-up, g/min	88.0	72.6	13.63	0.55	0.47
Eating while standing, % of total eating	96.0	97.8	0.594	4.12	0.06
Eating while walking, % of total eating	3.94	1.98	0.584	4.82	0.04
Drinking					
Water intake, L/d	3.13	3.11	0.033	0.14	0.71
Drinking time, min/d	1.70	1.76	0.471	0.01	0.93
Rumination					
Ruminating, h/d	10.6	9.73	0.292	4.07	0.06
Ruminating chews, no./d	56,069	49,847	1,415	8.27	0.01
Ruminating chewing rate, no./min	88.1	85.7	0.603	7.17	0.01
Pause between boluses, sec	5.83	6.75	0.177	12.03	0.002
Body posture					
Total standing, h/d	4.62	5.60	0.281	5.25	0.03
Total walking, min/d	13.9	13.4	0.844	0.13	0.72
Total lying, h/d	18.4	18.1	0.171	1.65	0.21

Lying idling, h/d	7.81	8.37	0.321	1.32	0.26
Lying idling, % total idling	82.7	88.2	1.226	8.78	0.01
Lying down frequency, no./d	24.2	26.9	3.555	0.24	0.63
Body maintenance					
Defecating, min/d	1.37	0.64	0.222	4.57	0.05
Urinating, min/d	1.77	1.43	0.253	0.75	0.40
Scratching, sec/d	99.0	93.0	0.246	0.07	0.79
Total rubbing, sec/d	352	218	0.901	2.63	0.12
body, $(sec/d+1)^{0.25}$	3.17	2.59	0.310	1.54	0.23
sec/d	99.5	44.1			
head, $(sec/d+1)^{0.21}$	2.68	2.13	0.170	4.33	0.05
sec/d	108	35.6			
Other behaviours					
Yawning frequency, no./d	16.0	7.49	2.142	6.74	0.02
Pawing frequency, $no./d^0$	5.79	5.71	0.158	0.10	0.75
no./d	327	302			

‡ Superscripts indicate exponents in Box-Cox transformations.

Table 2-5. Effect of ammonia (NH_3) exposure on sheep (n = 12) eating behaviour in the feed palatability tests on days 10 and 11 when exposed to Ammonia and Control treatments

Variables	Control	NH_3	SEM	*F* value	*P* value
Alfalfa pellets					
Eating duration, sec	342	339	4.810	0.12	0.73
Mastication rate, chews/sec	2.28	2.20	0.012	18.0	0.00
Prehension biting rate, bites/sec	2.24	2.21	0.120	0.03	0.87
Sorghum chaff					
Eating duration, sec	307	299	13.68	0.17	0.68
Mastication rate, chews/sec	2.28	2.26	0.018	0.55	0.47

Table 2-6. Effect of ammonia (NH_3) exposure on sheep (n = 12) faecal cortisol and corticosterone metabolites concentrations and respiratory rates on days 3 and 6 and rectal temperature on days 3, 6 and 11 when exposed to Ammonia and Control treatments

Variables	Control	NH_3	SEM	*F* value	*P* value
Cortisol metabolites, day 3, $1/\sqrt{}$(ng/g)	0.087	0.080	0.003	1.63	0.21
ng/g	123.5	156.3			
Cortisol metabolites, day 6, ng/g	146.2	157.5	16.12	0.22	0.64
Corticosterone metabolites, day 3, ng/g	183.0	204.8	9.249	2.48	0.13
Corticosterone metabolites, day 6, ng/g	187.2	234.8	14.81	4.63	0.04
Respiratory rate, no./min	63.3	80.1	1.836	37.36	0.00
Rectal temperature, ℃	38.76	38.81	0.028	1.39	0.25

Table 2-7. Effect of ammonia (NH_3) exposure on sheep (n = 12) adjusted latency score to approach ambiguous locations in the cognitive bias tests on day 14 and day 1 of the following period when exposed to Ammonia and Control treatments

	Control	NH_3	SEM	*F* value	*P* value
Adjusted latency score to approach M[1]	77.1	85.1	6.931	0.58	0.46
Adjusted latency score to approach AP[1], ln	2.91	3.01	0.261	0.06	0.81
Adjusted latency score to approach AP	18.4	20.3			

[1] M = a middle ambiguous location (1.0 m far from the positive location), AP = a partially positive ambiguous location (0.5 m far from the positive location)

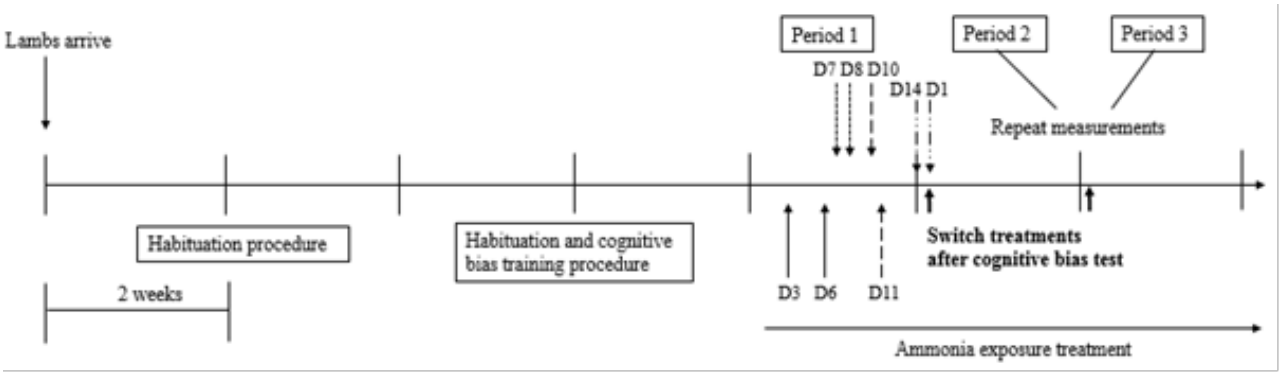

Figure 2-1. Timeline of the experimental procedures. (D3 and D6: faecal cortisol or corticosterone metabolites measurement; D3, D6 and D11: rectal temperature measurement; D1-D14 except D7 and D8: respiratory rate measurement; D7 and D8: general behaviour recording; D10 and D11: feed palatability test; D14 and D1 of the following period: cognitive bias test; D1 of periods 2 and 3: treatments changed after cognitive bias test).

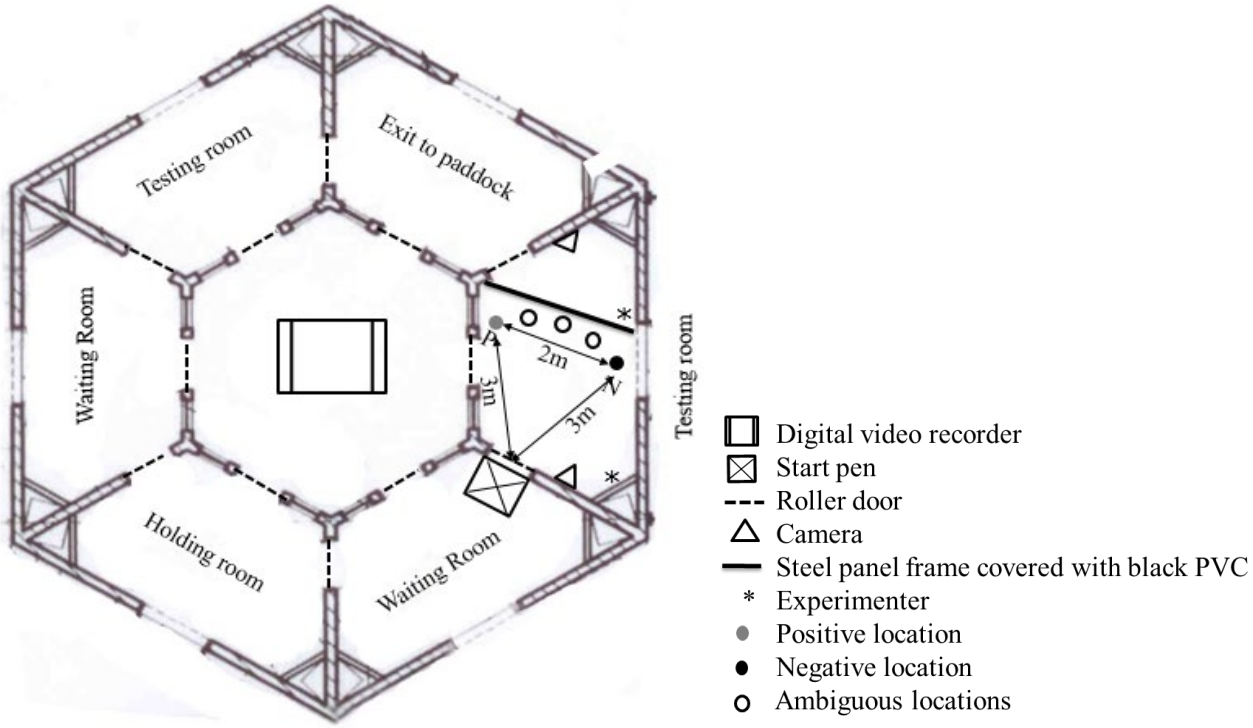

Figure 2- 2. Diagram of the cognitive bias training and test facility and positions of ambiguous buckets for cognitive bias testing, set up for a “left side positive” trained sheep.

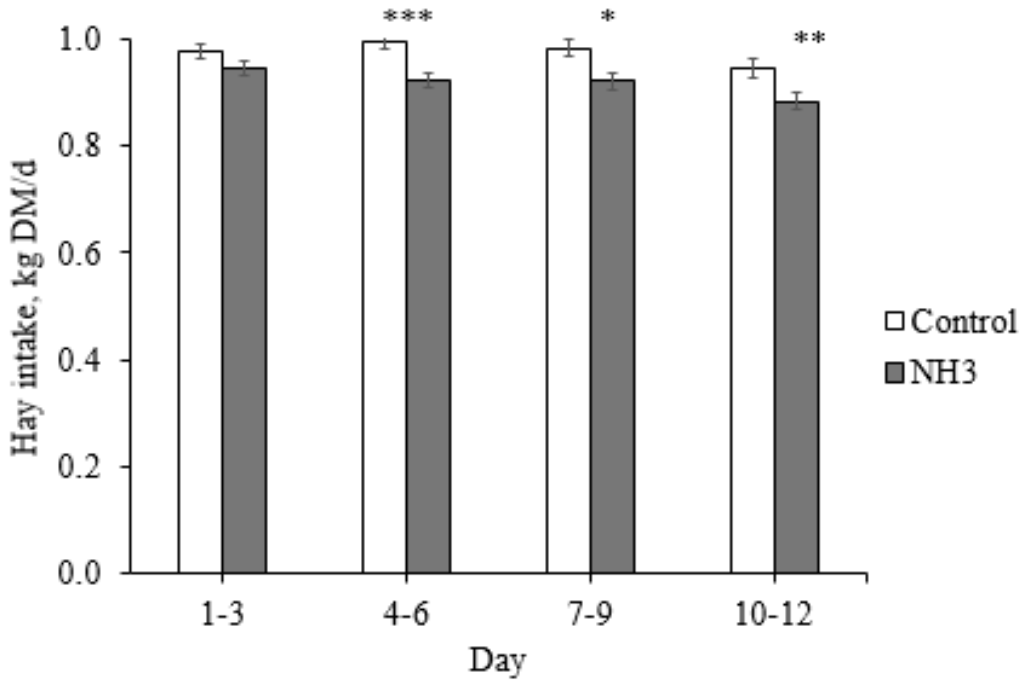

Figure 2- 3. Effect of ammonia (NH_3) exposure on hay DMI of sheep (n = 12) exposed to Ammonia and Control treatments during a 12-d period. *** = $P < 0.001$, ** = $0.001 < P < 0.01$, and * = $0.01 < P < 0.05$ represent hay DMI differences between treatments.

3 Chapter 3: Climatic influences on the mortality of sheep during long-distance sea transport

3.1 Abstract

Research on the causes of sheep death in sea voyages from Australia to the Middle East is limited, in particular little is known about the influence of climatic factors. Mortality data from 417 shipments of sheep exported over an 11-year period (November 2004 to June 2015) were modelled retrospectively to determine the key correlates. The statistical analysis were performed for both the full dataset with 417 voyages based on actual and estimated departure and arrival dates and the restricted dataset with 71 voyages based on actual dates. Results of the full dataset demonstrate a seasonal mortality pattern, with more deaths occurring on sea voyages leaving Australia in the southern hemisphere winter or spring than those departing in Australian summer or autumn. Heat stress and inadequate fat mobilization for energy supply when sheep are inappetant on ships may explain this seasonality. Based on the two models, the voyage and weather factors associated with sheep mortalities include departure year, autumn departure season in the southern hemisphere, voyage duration, single or multiple loading port(s), weekly mean dry bulb temperature and mean wind speed at the departure ports, and weekly mean humidity at the destination ports. Significant correlations were observed between weather variables at the departure ports in the Australian winter and a high sheep mortality rate during voyages. This, together with the heat the anticipated increased heat stress risk as a result of climate change, suggests that there could be review of the trade from Australia in the southern hemisphere winter. The influence of weather at the departure ports should be considered in sheep mortality prediction models, especially Australia's heat stress risk assessment model.

Keywords: climate, mortality, season, sea transport, sheep, weather

3.2 Introduction

Live sheep export from Australia to the Middle East (mostly Merinos and first-cross wethers, Deards, *et al.* 2014) is the largest live animal trade by sea globally. In 2017, 1.74 million head of live sheep were loaded and 12 377 head died (DAFF 2018). Mortality is the top welfare issue related to sea transport of livestock cited by veterinarians, livestock exporters, ship owners, and animal scientists (Pines, *et al.* 2007), which has attracted consistent attention. Early research demonstrated that sheep mortality rates during voyages to the Middle East were higher on shipments leaving Australia in the second half of the year (Higgs, *et al.* 1991). Inappetence was believed to be the primary cause of mortality of sheep exported from Australia in August (Richards,

et al. 1989), associated with inadequate fat mobilization for energy supply during inappetance, following good grass availability in south-west Australia at this time (Higgs, *et al.* 1991). Thus pre-embarkation conditions affect on-board mortality, but it is not known if this includes weather.

Heat stress is another major cause of mortality on shipments (Caulfield, *et al.* 2014). A Heat Stress Risk Assessment model (HSRA) is included in Australian Standards for the Export of Livestock (DAFF 2011) to predict mortality and reduce stocking density on ships as necessary. This model estimates the heat stress risk by predicting sheep mortality based on the distribution of wet bulb temperature (T_{WB}) at the destination or en route for the specific time of year. The prediction is adjusted for sheep factors, including liveweight, body condition, coat type and acclimatisation zone, and ship factors such as ventilation and stocking density on board shipments (Ferguson, *et al.* 2008). However, the climatic conditions at the departure ports are not taken into consideration by HSRA, and their roles in sheep mortality prediction are unknown.

Therefore, this study aimed to explore the roles that climatic conditions at the departure ports and voyage factors play in predicting sheep mortality occurrence during live export from Australia to the Middle East, based on 417 voyage records over an 11-year period (November 2004 to June 2015).

3.3 Materials and methods

3.3.1 Sources of data

Voyage information and reported sheep mortality on shipments from Australia to the Middle East (2004-2015) were derived from Australian Government sources (DAFF 2018). Based on the 418 voyages summarized, seven outliers were selected by creating a box plot, to determine if there were extraneous, non-weather related causes. According to the record of high mortality voyages by RSPCA Australia (2018), only one outlier, with mortality apparently arising from mechanical issues and a change of feed, had been omitted from our selection. We therefore excluded this voyage record. Inanition related to heat stress or/and salmonellosis were the stated reasons for high mortality rates in the other voyages, and one was not recorded.

A total of 417 voyages occurring in all seasons, carrying 30.7 million head of live sheep were utilised for this study. During these voyages, the mean number of sheep loaded was 73 629 ± 19768 head (SD), range 1388 to 119753 head; 265 999 sheep mortalities occurred, producing an overall average mortality rate of 0.86% ± 0.51% (SD), range 0.13% to 5.53%. Voyage duration was determined as the number of days from the first sheep loaded until the last sheep was unloaded,

which was on average 23 ± 5 days (SD), range 11 to 39 days. It usually takes 1-2 days for sheep to be loaded or unloaded at each departure or destination port (Dawn Lowe, personal communication; MLA 2015b).

Reports for 71 voyages included the actual departure and arrival dates, and the remaining 346 were estimated from the published record of the departure or arrival month of each voyage (DAFF 2018). If the voyage departed from Australia and arrived in the Middle East within the same month, arrival and departure dates were estimated as the middle day of the month ± (0.5 x voyage duration), respectively. If the voyage departed and arrived in adjacent months, arrival and departure dates were estimated as the last day of the first month ± (0.5 x voyage duration). The week around the relevant actual or estimated departure or arrival date of each voyage was determined by projecting the date forward and backward by 3 days.

Weather data at the departure ports in Australia were acquired from on-line weather records (Weather Underground 2018) through searching the relevant dates within the departure week and local weather station(s) as shown in Table 3-1. Due to a lack of record of weather data at Portland port before October 2013 at Mount Gambier Airport weather station, these data were acquired from Melbourne Airport weather station. Mean values were calculated if the voyage departed from two or three different ports. Weather data at single destination ports were obtained from an on-line database (Animals Australia 2018), through searching the relevant dates within the arrival week and destination country. For the shipments discharged at multiple destination ports, weather data of the largest importing country that year was used (Table 3-2). Taking into account the variable time spent in loading or unloading sheep at each port of multiple loading or destination ports, instead of using daily weather data, weekly mean values of weather records at departure or destination ports were used for analysis.

In order to investigate the impact of forecast bias in predicting the weather conditions at destination ports or en route on sheep mortality prediction before export, each destination weekly mean dry bulb temperature (T_{DBm}) was adjusted by adding an absolute forecast error of ± 4.2°C to produce T_{DBmadj}. This was determined from a typical error in 10-day mean air temperature forecasts in Russia (Vil'fand, *et al.* 2010).

3.3.2 Statistical analysis

Statistical analysis was carried out in Minitab 17.0 (Minitab Version 17; Minitab Inc., State College PA, USA), based on two datasets: one of 417 voyages with both actual and estimated departure and

arrival weekly data (the full dataset); the other one including 71 voyages with actual departure and arrival weekly data (the restricted dataset). After using smoothed lines of time series plots of each voyage or weather variable against mortality rate to check for non-linear trends, we selected best-subset linear regression analysis to identify the most useful variables in predicting sheep mortality rate. To achieve normal distribution of the residuals after fitting regression models, the mortality rate data (as decimals) were transformed to $\log_{10}$ values, because the mortality rates during these voyages were contained in a narrow range within possible values (0.13% to 5.53%). In the best-subset selection, log10 transformed mortality rate was used as the response variable. Twenty independent variables considered in the best-subset selection are summarized in Table 3-3. A statistical description of sheep mortality rates on the selected voyages and weather at departure and destination ports is shown in Tables 3-4 and 3-5, respectively. The maximum number of independent variables that can be included in the model is 20. The method allows determination of models with as few predictors as possible based on the Mallows' Cp (Mallows 1973) and Akaike Information Criterion (AIC) (Akaike 1973). A small value of Mallows' Cp and AIC indicated that the relevant subset model was relatively precise with least variance in estimating the true regression coefficients and predicting future responses. Extra precision was not obtained by adding more predictors. Mallows' Cp values greater than the number of predictors selected indicated that the relevant models had a poor fit. Analysis was performed to select the best subset to fit regression model, with the number of sheep loaded as a weighting factor. Adjusted R^2 ($R^2_{(adj)}$) and standard error of the regression (S) were used as fit statistics. The model with the highest $R^2_{(adj)}$ was assumed to be the model with the lowest error mean square. A variance inflation factor (VIF) was used to evaluate how much the variance of the estimated regression coefficients was inflated, which might occur if the predictor variables were linearly related. Regression coefficients were assumed to be not correlated when VIF < 1, moderately correlated when 1 < VIF < 5, and highly correlated when VIF > 5 to 10. VIF values greater than 5-10 suggest that the regression coefficients are poorly estimated due to multicollinearity (Minitab 17 Support 2018). One-way ANOVA was performed to assess the seasonal effect on sheep mortality rate at a significance level of 0.05, using log10 transformed mortality rate (as decimals) as the dependent variable.

3.4 Results

Compared with the full dataset over the 11-year period, the restricted dataset was only based on records over 5 years, which demonstrated lower mean or maximum mortality rate values and smaller ranges of mortality rates except in 2006 or in the southern hemisphere summer (Table 3-4). Compared with the 7.7% of voyages that had multiple exporters in the full dataset, only 1 voyage in the restricted dataset had multiple exporters. Similarly, the full dataset had a higher percentage of

voyages (34.3%) with sheep loaded at multiple ports than the restricted dataset (22.5%). For both datasets, sheep for 97% of voyages were loaded at Fremantle port in Western Australia and/or Adelaide port in South Australia. The full dataset also had a higher percentage of voyages (80.1%) with sheep discharged at multiple destination ports than the restricted dataset (59.2%). Compared with the full dataset, voyages in the restricted dataset generally had lower mean or maximum mortality rate values and smaller ranges of mortality rate, except the voyages with multiple exporters or multiple loading ports or departing from Victoria and Tasmania or Victoria and Western Australia, which had higher mean mortality rates.

As shown in Table 3-5, T_{DBm} was higher at the destination than departure ports in both datasets, but there was considerable range. Weekly mean humidity (H_m) was greater at departure than destination ports in both datasets, but differed less between the departure and destination ports in the restricted dataset. Wind speeds were similar between departure and destination ports. The weekly mean values of weather variables were similar in the two datasets, except that the minimum and maximum values were generally less extreme in the restricted dataset.

Based on the full dataset, there was a significant effect of departure season on sheep mortality rate as determined by one-way ANOVA (F (3, 413) = 24.45, P <0.001) (Table 3-6), with higher sheep mortality rate on the voyages leaving from Australia in the southern hemisphere winter or spring than those departing in Australian summer or autumn. In the restricted dataset, season was not significantly related to sheep mortality rate (F (3, 67) = 2.46, P = 0.07).

Best-subset linear regression analysis revealed that no single variable could accurately predict sheep mortality rate. Log_{10} transformed sheep mortality rate were regressed on two best subsets derived from the full dataset (n = 6, Mallows' Cp = 4.8, AIC = -1321.2) or the restricted dataset (n = 4, Mallows' Cp = -0.2, AIC = -242.2) respectively, as demonstrated by the full model ($R^2_{(adj)}$ = 25.8%, S = 54.5) and the restricted model ($R^2_{(adj)}$ = 35%, S = 48.7) in Table 3-7. Voyage duration and departure weekly T_{DBm} entered these two models as common predictors, and there was a trend for multiple loading ports to be included in both models. Sheep mortality rate increased with voyage duration, but decreased when using multiple loading ports. Departure weekly T_{DBm} was the only common weather variable included in both models, with increased mortality at low departure temperatures in Australia (i.e. in the southern hemisphere winter), which is consistent with the seasonal mortality pattern. Unlike the full model, the restricted model selected destination H_m, demonstrating that the higher the destination H_m, the greater the sheep mortality rate. The full model included an extra 3 variables (departure year, autumn departure season in the southern

hemisphere, and departure weekly wind speed (WS_m)) compared to the restricted model. Sheep mortality rate decreased with advancing departure year, and increased with higher departure weekly WS_m. Departure in the southern hemisphere autumn was associated with a reduced sheep mortality rate.

3.5 Discussion

Results based on the full dataset demonstrated a seasonal mortality pattern in sheep exported by sea from Australia to the Middle East, with higher mortality rate during voyages leaving Australia in the southern hemisphere winter or spring than those departing in Australian summer or autumn. This is consistent with early research demonstrating that higher mortality rate occurred when shipping sheep to the Middle East in Australian August (winter) than those shipped in Australian May (autumn) (Richards, *et al.* 1989). In the restricted dataset, the effect of departure season on sheep mortality rate was not significant, which may be because this dataset did not have a large enough range of seasonal measures in just 5 years, compared with 11 years for the full dataset. Compared with other departure seasons, export in the southern hemisphere autumn plays an important role in reducing sheep mortality rate, as demonstrated by the significant negative correlation between these two factors in the full model. We consider several possible explanations for this seasonality of sheep mortality.

3.5.1 Climatic differences between departure and destination ports

Both models found that mortality rate was negatively correlated with departure weekly T_{DBm}, corresponding to a higher mortality rate on voyages departing in the Australian winter and arriving in the Middle Eastern summer. In the Australian winter, there are major climatic differences between the departure ports, in the southern hemisphere, and the destination ports, in the northern hemisphere. Environmental conditions on shipments departing in the southern hemisphere winter can become extremely hot and humid with little or no diurnal variation in ambient temperature after crossing the equatorial regions and entering into the Middle East (Norris, *et al.* 2003). Sheep may leave Australia in winter at 0°C and arrive in the northern hemisphere in summer with temperatures of > 40°C (Pal & Eltahir 2016). Sheep are expected to be exposed to heat stress from the temperatures experienced at the destination ports and during the voyage (Phillips 2016). This is demonstrated by the maximum temperatures at the destination ports during the selected voyages, which at 41.5°C are well above the sheep's upper critical temperature (Alexander 1974). When air temperature exceeds sheep body temperature (normally 38.3-39.9°C), heat stress occurs because the cooling effect of ventilation is reduced or eliminated, even for voyages with reduced stocking density (Mahjoubi, *et al.* 2015).

Early research proposed that increased mortality of sheep exported from Australia in August was because the initial fat mobilization for energy supply did not persist, as evidenced by reduced plasma concentrations of non-esterified fatty acid (NEFA), beta-hydroxybutyrate and glycerol compared to sheep exported in May (Richards, *et al.* 1991). Instead, increased plasma protein and urea concentrations in sheep exported in August suggest that endogenous protein was supplying alternative energy. This may be related to heat stress that sheep confronted after the ship crossed the equatorial regions and entered the Middle Eastern summer, since adipose tissue mobilization is apparently reduced in heat-stressed sheep, as evidenced by low NEFA concentrations in two studies (Sano, *et al.* 1983; Mahjoubi, *et al.* 2015), although not in the work of Alhidary, *et al.* (2012). There is a variety of factors, including age of the sheep, duration and severity of heat stress, and blood sampling time, that may explain this discrepancy of results.

The Australian government investigates all incidences of sheep mortality greater than 2%, but the contribution of heat stress to sheep mortalities during live export may have been underestimated due to their overestimation of the heat stress threshold and because post mortem signs of heat stress may be confused with salmonellosis (Phillips 2016). Heat stroke causes multi-organ failure, including myocardial infarction, renal tubular necrosis and nephrosis and acute liver necrosis (Quinn, *et al.* 2014). Lambs that have died of heat stress present with gross lesions, acute renal swelling and pallor, muscle pallor, and chronic bronchointerstitial pneumonia (Sula, *et al.* 2012). Renal dysfunction has been detected in sheep exposed to prolonged high heat and humidity, similar to that experienced by sheep during export from Australia to the Middle East (Stockman, *et al.* 2011). Since most sheep are exposed to Salmonella spp. in the feedlot and/or the voyage, it is speculated that the combination of heat stress and persistent inappetence-salmonellosis-inanition may increase sheep mortality in live export (Phillips 2016).

Destination weekly H_m included in the restricted model demonstrates the difficulty in successfully dissipating metabolic heat in a humid environment (Thwaites 1985). In the Gulf region, the absence of clouds, high incoming solar radiation, low reflection of heat by the sea, high evaporation rate increasing water vapour and topographical enclosure of the sea magnifies the humidity in this region (Pal & Eltahir 2016). The reason why the destination H_m was only included in the restricted model may be because humidity at the destination ports is highly variable, as has been measured on ships travelling to the Gulf region (Zhang, *et al.* 2017). The use of actual arrival dates in the restricted dataset would have captured humidity values more accurately.

In sheep, the lower critical temperature is influenced by the thickness of the fleece. Australian Standards for the Export of Livestock (DAFF 2011) requires that sheep must have a wool length not more than 25 mm at the time of shipment or have been freshly shorn in the 10 days before export. The cold tolerance (environmental T_{DB}) of freshly shorn adult sheep with 7 mm of fleece in dry windy weather (7 m/s wind, close to the maximum WS in both datasets) would be -11°C, but, when wet and exposed to a 7 m/s wind, a similar sheep could withstand only +14°C (Alexander 1974). Thus, the minimum temperatures at the Australian ports are well above the lower critical temperature for sheep in dry weather. However, as the main port for Australian sheep export, Fremantle port in Western Australia has a Mediterranean climate with warm to hot dry summers and mild wet winters, with most precipitation falling in the winter months. The sheep are kept in sheds for about a week before loading to acclimatise to the conditions. Under wet, windy conditions in other departure ports in the Australian winter, where no sheds are used, there is likely to be increased convective heat loss from the body to the environment (McArthur & Monteith 1980), resulting in colder conditions being experienced by the sheep than in still-air (the wind-chill effect, Ames & Insley 1975).

The high mortality rates in exports with low departure T_{DBm} and high departure WS_m suggest that inclement weather in the Australian winter is adversely affecting sheep tolerance of heat stress. The colder temperatures at the departure ports appear to be preventing acclimatisation to the higher temperatures later. This could be due to skin thickening in the cold temperatures, which later restricts heat loss. Cold-stressed sheep can increase skin thickness within two hours of cold exposure, reaching a maximum effect after 2 weeks (Wodzicka-Tomaszewska 1960). The reason why the departure weekly WS_m was only included in the full model may be because the minimum and maximum values of weekly temperature, humidity and wind speed were less extreme in the restricted dataset.

3.5.2 Seasonal variation in pasture availability and tissue mobilization patterns

Ruminants deposit adipose tissues to store energy when the feed resources are adequate, and mobilize fat to meet energy demands during periods of feed shortages (Ørskov 1998). In the Mediterranean regions of Australia, the quality and quantity of feed resources reach a nadir in autumn (McKeown & Smith 1970). However, unlike the fat mobilization failure in the inappetant sheep exported from Australia in August (winter), inappetant sheep on voyages leaving Australia in May (late autumn) could mobilize fat reserves for energy supply persistently, as evidenced by increasing concentrations of plasma NEFA, beta-hydroxybutyrate and glycerol (Richards, *et al.* 1991). This may explain why the full model identified the autumn departure season in the southern

hemisphere as a low mortality period for Australian live sheep export. It is speculated that immature wether sheep exported in the southern hemisphere autumn may be adjusted to mobilising adipose reserves for energy supply when inappetant, as a consequence of being in the later stages of body tissue depletion after prolonged nutrient shortages in summer (Purser 1980; Ball, *et al.* 1997). High mortality is also more likely to occur in sheep from zones with a longer pasture-growing season (Higgs, *et al.* 1999). The reasons why the autumn departure season in the southern hemisphere was only included in the full model may be because: (1) the restricted dataset did not have a large enough range of seasonal measures in just 5 years, compared with 11 years for the full dataset. (2) The smaller ranges of sheep mortality rates in the restricted dataset did not capture extreme mortality shipments, i.e. above 2.36%. (3) According to the mean monthly temperature in autumn in Fremantle from 2004 to 2015 (Table 3-8, Weather underground 2018), autumns over those 5 years in the restricted dataset were generally warmer than the rest of years included in the full dataset, perhaps supporting increased grass growth. This may indicate that sheep were not mobilising body tissue as much on the restricted selected voyages, if they had not been underfed before being loaded on board. (4) The restricted model also included the destination weekly humidity, which may mask the seasonal temperature effects.

3.5.3 Photoperiod change and tissue mobilization patterns

Although nutrition generally overrides photoperiodic influences on tissue mobilization (Martin, *et al.* 2002), it might be expected that an abrupt change from a short daylength in the southern hemisphere winter to 24 h of light/day on board (Lynn Simpson, personal communication) would promote adipose tissue mobilization in sheep. A similar phenomenon has been reported in ewes transferred from 9 h to 16 h of light/day (Bocquier, *et al.* 1998). However, the continuous light on board may fail to activate a long-day response, as has been found during the northern hemisphere summer (20 h light/day) at high latitudes (Ebling, *et al.* 1988; Wagner, *et al.* 2007), maybe because melatonin secretion is disrupted (Ebling, *et al.* 1988). However, in the southern hemisphere autumn, adipose tissue mobilization may be increased by the transition from a declining daylength to the constant light on board, as suggested by evidence of the weight loss in cattle caused by the supplementary light during decreasing daylength (Phillips, *et al.* 1998).

3.5.4 Other key correlates of sheep mortality on sea voyages

Departure year

The declining sheep mortality rate from 0.96% in 2005 to 0.62 % in 2015 can be attributed to a number of ongoing management changes (Norris & Norman 2005). First, the Australian Standards for the Export of Livestock (DAFF 2011) came into effect in 2005, which made government

responsible for the regulation of the livestock export process. Second, demand in the Middle East market has changed from larger, older sheep, to an increasing emphasis on quality, a young age and leanness. Young wethers (i.e. lambs) have a lower mortality rate than adult sheep, because of a stronger appetite driven by the overriding demands of tissue growth (Higgs, *et al.* 1991). This might be also because young sheep are more likely to mobilise fat tissue in response to feed shortages (Drew & Reid 1975; Aziz, *et al.* 1992), and particularly subcutaneous fat (Little & Sandland 1975; Aziz, *et al.* 1992). Together with a higher surface area to volume ratio in lambs, this may have protected them against heat stress (Sykes, *et al.* 1976). Third, the time that sheep spend in the export system has reduced and it is probably less stressful because of faster ships with better ventilation systems. The reasons why the departure year was only included in the full model may be because the limited 5-year record in the restricted dataset did not capture enough influence of the management changes described above on sheep mortality rate, compared to the 11-year record in the full dataset.

Voyage duration

The longer voyage duration, the higher mortality rates occurred. This may relate to the higher mortality rate measured on shipments departing from Victoria than WA (Kelly 1995), but it could also be because of a difference at assembly depots. Sheep resistance to infection, in particular the Salmonella spp. which present an increasing challenge over the voyage duration, may decline over time, contributing to increased inanition in the later stages of the voyage (Higgs, *et al.* 1993). Government reviews of high mortality voyages (>2% mortality) does not take into account the time and mortalities when sheep were transported before loading and after discharging. Adjusted mortality rates, corrected for the voyage duration (Madin 2015), should be preferred in future investigation.

Single or multiple exporters

The decreased mortality risk on sea voyages with multiple loading ports may indicate that sourcing sheep from different regions might help decrease the mortality risks. This is consistent with a previous spatial analysis study demonstrating higher mortality risk in sheep coming just from the high rainfall zones with a longer pasture-growing season in the south-west region in Western Australia, from where most sheep are shipped to the Middle East (Higgs, *et al.* 1999). The longer pasture growth means that the sheep are likely to be in fatter condition, whereas if multiple loading ports are involved, sheep are likely to also come from the lower rainfall zones such as the eastern states. These sheep will be thinner, not least because of their longer journey by truck to Fremantle, and therefore more likely to have a good appetite to force their way to the feed.

3.5.5 Implications for Australian Heat Stress Risk Assessment model

Several relationships between climatic variables and mortality highlighted in this study suggest that refinement of the Australian HSRA could reduce sheep mortality during live export. Firstly, the strong association between sheep mortality rate and temperature at the departure ports suggests the inclusion of weather at the departure ports in the HSRA model, based on the actual weather rather than predicted. Secondly, recognising the seasonal mortality pattern, the HSRA should be reviewed to examine sheep export from southern Australian ports such as Portland and Adelaide port in the southern hemisphere winter, which is already restricted for the export of *Bos taurus* cattle (DAFF 2011). In particular, due to climate change, summer temperatures in the Arabian Gulf are apparently increasing more rapidly than in winter. A relatively steep increase in the daytime maximum temperature in summer and the number of heatwave days is expected, which impact would be exacerbated because of the increased daily minimum night-time temperature and reduced night-time radiative cooling (Lelieveld, *et al.* 2014). According to Pal & Eltahir (2016), the 60°C threshold of annual maximum daily temperature in Kuwait will be frequently exceeded several times by the end of the century, and maximum daily temperature at 50°C will become normal during July, August, and September. Thirdly, due to the climate change, the range of climatic parameters used for the HSRA needs to be adequate to cover possible weather conditions in the Middle East, and allow sufficient prediction for occurrence of severe heat stress. This situation may be more severe if considering the date of annual Eidh al-Addha festival, as the major market for sheep export to Saudi Arabia, which will be in mid-summer when temperatures are at their peak over the next decade (Zhang, *et al.* 2017).

3.5.6 Limitations

There are several limitations to this study. Firstly, due to a lack of discharge details, the major weather differences between multiple destination ports of the voyages, particularly humidity differences, were not considered in the analysis. Secondly, the weather and other stressors en route were not considered due to a lack of data. The ship Master's reports have details of weather data recorded en route, but these are not released for analysis. Thus, the relevance of the measurements at the departure and destination ports in relation to the conditions en route could not be compared. Thirdly, voyage duration and mortality rates obtained from Australian Government do not include the time and mortalities when sheep were transported before loading and after discharging (the latter estimated as 3%, Scharp 1992). Fourthly, the absolute forecast error we used for destination T_{DBm} adjustment was obtained from the 10-day air temperature forecasts in Russia. There may be differences in this error between the Middle East and Russia, which were not taken into account. For voyages over 10 days, this adjustment may overestimate the forecast accuracy, since 11-15 days

forecasts have 30% more absolute errors in the Global Forecast System than the 6-10 days forecasts (WDT 2018). Finally, the impact of climate change in the Middle East was not included in the analysis.

3.6 Conclusions

Based on the full dataset, this study demonstrated that sheep exported to the Middle East from Australia in the southern hemisphere winter or spring have substantially higher mortality rates than those shipped in Australian summer or autumn. The key correlates of sheep mortality identified in this study suggested that exporters could reduce the mortality risk through either preferentially exporting sheep in the Australian summer or autumn whenever possible, or better prediction of high mortality shipments based on a refined Australian Heat Stress Risk Assessment model. This study also suggested that the seasonal mortality pattern may be linked to heat stress during the voyage, tissue mobilization patterns, and photoperiod history sheep experienced prior to the export, but further research is required to address the possible roles of these factors.

3.7 Tables and figures

Table 3-1. Location of weather stations used to obtain climatic data at the departure ports

Departure port	State/territory[1]	Weather station	Coordinates
Fremantle	WA	Perth International Airport	31.9° S, 116.0° E
Broome	WA	Broome International Airport	17.95° S, 122.23° E
Geraldton	WA	Geraldton Airport	28.80° S, 114.70° E
Hedland	WA	Port Hedland International Airport	20.37° S, 118.63° E
Portland	VIC	Mount Gambier Airport	37.75° S, 140.77° E
	VIC	Melbourne Airport	37.67°S, 144.85°E
Adelaide	SA	Adelaide International Airport	35.0° S, 138.5° E
Devonport	TAS	Devonport Airport	41.17° S, 146.43° E
Darwin	NT	Darwin International Airport	12.42° S, 130.89° E

[1] WA = Western Australia; VIC = Victoria; SA = South Australia; TAS = Tasmania; TN = Northern Territory.

Table 3-2. Top three Middle Eastern importers for Australian live sheep export from 2004 to 2015, as determined by the numbers of sheep imported (MLA 2014, 2016)

Year	Biggest importer	Second-biggest importer	Third-biggest importer
2004	Kuwait	Jordan	Bahrain
2005	Saudi Arabia	Kuwait	Jordan
2006	Saudi Arabia	Kuwait	Jordan
2007	Saudi Arabia	Kuwait	Bahrain
2008	Kuwait	Saudi Arabia	Oman
2009	Kuwait	Bahrain	Saudi Arabia
2010	Kuwait	Bahrain	Qatar
2011	Kuwait	Qatar	Bahrain
2012	Kuwait	Qatar	Jordan
2013	Kuwait	Qatar	Jordan
2014	Kuwait	Qatar	Jordan
2015	Kuwait	Qatar	Bahrain

Table 3-3. Independent variables included in the best subset selection analysis

Voyage variable	Weather variable
Departure Year	Departure weekly mean dry bulb temperature
Departure week within the year[1]	Departure weekly mean dew point
Departure in spring[2]	Departure weekly mean humidity
Departure in summer[2]	Departure weekly mean wind speed
Departure in autumn[2]	Destination weekly mean dry bulb temperature
Number of sheep loaded	Destination weekly adjusted mean dry bulb temperature[6]
Voyage duration	Destination weekly mean wet bulb temperature
Single or multiple exporter(s) [3]	Destination weekly mean dew point
Single or multiple loading port(s) [4]	Destination weekly mean humidity
Single or multiple destination(s) [5]	Destination weekly mean wind speed

[1] Coded from week 1 (1 January - 7 January) to week 52 (25 December - 31 December).

[2] Equal to 1 if it is spring or summer or autumn in the southern hemisphere and 0 if not. Three departure season variables all equal to 0 if it is winter in the southern hemisphere.

[3] Equal to 1 if it is multiple exporters and 0 if single exporter.

[4] Equal to 1 if it is multiple loading ports and 0 if single loading port.

[5] Equal to 1 if it is multiple destinations and 0 if single destination.

[6] A forecast error (± 4.2°C) was randomly added to each destination weekly mean dry bulb temperature.

Table 3-4. Descriptive statistics for sheep mortality rates (%) on the selected voyages categorized by voyage variables

	417 voyages					71 voyages				
Item	n	Mean	SD	Minimum	Maximum	n	Mean	SD	Minimum	Maximum
Departure year										
2004	6	0.63	0.20	0.40	0.85					
2005	39	0.96	0.54	0.26	2.66	10	0.76	0.42	0.32	1.69
2006	53	0.90	0.38	0.30	2.36	48	0.93	0.39	0.30	2.36
2007	47	0.94	0.56	0.13	2.53	6	0.47	0.23	0.13	0.82
2008	52	0.86	0.43	0.24	1.68	5	0.39	0.11	0.29	0.52
2009	48	0.94	0.37	0.31	1.93					
2010	37	0.91	0.56	0.36	3.32					
2011	29	0.81	0.43	0.26	1.94					
2012	32	0.85	0.54	0.27	1.92	2	0.61	0.18	0.48	0.73
2013	29	0.74	0.99	0.22	5.53					
2014	31	0.67	0.32	0.14	1.49					
2015	14	0.62	0.24	0.34	1.06					
Departure season in southern hemisphere										
Spring	108	0.96	0.39	0.24	2.06	20	0.83	0.34	0.32	1.46
Summer	105	0.72	0.39	0.13	2.36	19	0.86	0.53	0.13	2.36
Autumn	99	0.64	0.33	0.22	1.88	17	0.60	0.21	0.29	0.97
Winter	105	1.11	0.71	0.29	5.53	15	0.99	0.40	0.34	1.97

Voyages with single or multiple exporter(s)										
Single	385	0.88	0.52	0.13	5.53	70	0.82	0.41	0.13	2.36
Multiple	32	0.64	0.33	0.22	1.78	1	0.73	*	0.73	0.73
Voyages with single or multiple loading port(s)										
Single	274	0.82	0.44	0.13	2.66	55	0.77	0.34	0.13	1.69
Multiple	143	0.94	0.63	0.26	5.53	16	0.99	0.56	0.34	2.36
Voyages with loading ports in different states[1]										
SA	13	1.04	0.30	0.51	1.68	4	0.86	0.32	0.51	1.25
SA/WA	45	0.92	0.81	0.30	5.53	2	0.70	0.39	0.42	0.97
SA/WA/VIC	1	0.47	-	0.47	0.47	0				
VIC	9	0.63	0.23	0.27	1.03	1	0.56	*	0.56	0.56
VIC/SA	8	1.59	0.78	0.84	3.32	1	1.21	*	1.21	1.21
VIC/TAS	2	1.64	1.03	0.91	2.36	1	2.36	*	2.36	2.36
VIC/WA	78	0.87	0.45	0.26	1.97	12	0.90	0.46	0.34	1.97
WA	261	0.82	0.44	0.13	2.66	50	0.77	0.35	0.13	1.69
Voyages with single or multiple destination(s)										
Single	83	0.80	0.43	0.24	2.06	29	0.75	0.38	0.3	1.69
Multiple	334	0.87	0.53	0.13	5.53	42	0.86	0.42	0.13	2.36

[1] SA = South Australia; WA = Western Australia; VIC = Victoria; TAS = Tasmania.

Table 3-5. Descriptive statistics for weather variables at departure and destination ports based on the selected voyages

	417 voyages					71 voyages				
Variable [1]	n	Mean	SD	Minimum	Maximum	n	Mean	SD	Minimum	Maximum
Departure ports										
T_{DBm} (°C)	417	17.7	4.70	8.86	29.6	71	18.0	4.56	10.4	26.6
DP_m (°C)	417	9.34	2.94	-0.86	20.1	71	9.37	3.48	-0.86	16.0
H_m (%)	417	60.7	10.7	33.4	88.6	71	59.3	9.24	40.6	79.7
WS_m (m/s)	417	4.37	0.94	2.28	7.20	71	4.40	0.89	2.32	6.69
Destination ports										
T_{DBm} (°C)	415	26.5	8.83	6.63	41.5	71	25.1	8.63	10.3	41.4
T_{DBmadj}[2] (°C)	415	26.4	9.08	5.76	44.7	71	24.8	9.02	8.31	42.8
T_{WBm} (°C)	386	20.8	4.82	7.36	32.8	71	20.4	4.60	10.6	32.4
DP_m (°C)	415	8.05	6.43	-10.0	26.5	71	7.87	4.68	-1.06	20.3
H_m (%)	414	37.0	17.7	8.43	81.4	71	38.4	16.1	10.6	72.9
WS_m (m/s)	414	4.17	1.30	1.13	9.07	71	4.00	1.19	1.85	6.83

[1] T_{DBm}, T_{DBmadj} = mean, adjusted mean dry bulb temperature; T_{WBm} = mean wet bulb temperature; DP_m = mean dew point; H_m = mean humidity; WS_m = mean wind speed, as defined in the text.

[2] A forecast error (± 4.2°C) was randomly added to each destination weekly T_{DBm} record.

Table 3-6. One-way ANOVA for the effect of departure season on sheep mortality rate (in $\log_{10}$ base) during sea export

Mortality rate	Spring	Summer	Autumn	Winter
Full dataset with 417 voyages				
Mean mortality rate ($\log_{10}$ of decimal values) ± SEM	-2.06±0.019[a]	-2.21±0.023[b]	-2.24±0.020[b]	-2.02±0.024[a]
Mean mortality rate (%)	0.87	0.62	0.58	0.95
Restricted dataset with 71 voyages				
Mean mortality rate ($\log_{10}$ of decimal values) ± SEM	-2.12±0.042	-2.14±0.067	-2.25±0.038	-2.04±0.05
Mean mortality rate (%)	0.76	0.72	0.56	0.91

[a,b] Means with different superscript letters within a row are significantly different at $P < 0.001$.

Table 3-7. Best-subset linear regression models for sheep mortality rate (in $\log_{10}$ base) prediction based on the selected datasets

	Full model (417 voyages)			Restricted model (71 voyages)		
Items[1]	Coef[2]	*P*-value	VIF[2]	Coef	*P*-value	VIF
Constant	31.14	<0.001		-2.31	<0.001	
Departure year	-0.017	<0.001	1.05			
Southern hemisphere autumn[3]=1	-0.102	<0.001	1.09			
Voyage duration (day)	0.012	<0.001	1.91	0.023	0.002	2.46
Single or multiple loading port(s)[4]=1	-0.082	0.005	1.98	-0.141	0.008	2.33
Departure weekly T_{DBm} (°C)	-0.019	<0.001	1.44	-0.027	0.066	1.55
Departure weekly WS_m (km/h)	0.008	0.024	1.40			
Destination weekly H_m (%)				0.005	0.001	1.48

[1] T_{DBm} = mean dry bulb temperature; WS_m = mean wind speed; H_m = mean humidity, as defined in the text.

[2] Coef = coefficient; VIF = variance inflation factor.

[3] Equal to 1 if it is southern hemisphere autumn and 0 if not.

[4] Equal to 1 if it is multiple loading ports and 0 if single loading port.

Table 3-8. Descriptive statistics for monthly mean dry bulb temperature during Australian autumn (March-May) in Fremantle from 2004 to 2015

Departure year	Mean	SD	Minimum	Maximum
2004	27.3	4.16	24.0	32.0
2005	26.3	3.51	23.0	30.0
2006	25.7	5.51	22.0	32.0
2007	27.0	6.00	21.0	33.0
2008	23.3	4.16	20.0	28.0
2009	25.3	5.03	20.0	30.0
2010	25.0	5.57	20.0	31.0
2011	26.3	3.79	22.0	29.0
2012	25.0	5.00	20.0	30.0
2013	25.3	4.73	20.0	29.0
2014	25.7	5.86	19.0	30.0
2015	24.0	3.61	21.0	28.0

4 Chapter 4: Development of an effective sampling strategy for ammonia, temperature and relative humidity measurement during sheep transport by ship

4.1 Abstract

Ammonia, high temperature and high humidity have adverse effects on sheep during long distance live export from Australia to the Middle East, but none of these is effectively measured currently. On the basis of data maps obtained on two voyages of live sheep export, this study determined sampling strategies for ammonia, temperature and humidity measurement on the vessel. The difference between predicted High and Low ammonia sites on the shipment could be detected with five sampling sites of each. Margins of error were determined, which suggested that dry bulb temperature could be measured with six to eight sampling sites, but even twenty sampling sites were not sufficient to measure relative humidity. For the vessel recorded, considerably more ammonia sampling sites are required on closed decks than on open decks, with less variation for temperature measurement. The number of pens measured contributed more to the variance of ammonia and temperature measurement than the number of sampling sites within each pen on open decks. This study highlights the importance of a suitable sampling strategy to measure ammonia, temperature and relative humidity on board ship during live export.

Keywords: ammonia, live export, relative humidity, sampling strategy, temperature

4.2 Introduction

As the world's largest exporter of live sheep, Australia exported 2.18 million live sheep in 2014-15, with 97% transported to the Middle East (MLA 2015a), nearly all by sea in voyages lasting 10-26 days (Phillips 2008).

During such long duration transportation at high stocking density, ammonia (NH_3) is released from animal excreta, which accumulates on the pen floor during the voyage and is not cleaned out until after discharge of the animals at the destination port. Ammonia exposure has detrimental effects not only on human health in various workplaces, but also on animal health, growth and production performance (De Boer & Morrison 1988; Gustin, *et al.* 1994; Cole, *et al.* 2000; Kristensen & Wathes 2000; Costa, *et al.* 2003; Phillips, *et al.* 2010, 2012a). Under simulated live export conditions, NH_3 adversely affected the welfare of steers and sheep by inducing inflammation of the respiratory system and reducing feed intake and body weight gain (Phillips, *et al.* 2010, 2012a). Therefore, not only stakeholders in the industry have identified NH_3 as one of the top five welfare issues (Pines, *et al.*

2007), but also researchers have hypothesised it to be related to one of the main causes of sheep mortality, inappetence (Richards, *et al.* 1989).

So far, there is no universally applied maximum NH_3 concentration for live export. The current exposure threshold value for NH_3 exposure (25 ppm, 17 mg/m^3) recommended for the Australian live export industries (NOHSC 1995; MLA 2001; Costa, *et al.* 2003; Stacey 2003) is based more on human safety limits suggested as a time weighted average exposure level (the average exposure during an 8-h day) than on animal welfare. Despite this, a critical concentration of 30 ppm (21 mg/m^3) NH_3 has been suggested for steers based on the physiological responses to high NH_3 exposure for 12 days under simulated live export conditions (Phillips, *et al.* 2010). Similar research with sheep suggested that the critical concentration should be the same as proposed for steers (30 ppm, 21 mg/m^3), but with limited evidence (Phillips, *et al.* 2012a). Due to a lack of enforced regulation of NH_3 concentrations, studies on livestock voyages from Australia to the Middle East have found some NH_3 concentrations to be above the recommended maximum, 25-30 ppm (17-21 mg/m^3) (MLA 2001; Tudor, *et al.* 2003; Pines & Phillips 2011). The most comprehensive investigation conducted by Pines & Phillips (2011) showed the concentrations at most of their measurement locations were below 18 ppm (13 mg/m^3), however, several sites were above 25 ppm (17 mg/m^3), in particular those parts of the ship with insufficient ventilation and/or high temperatures and humidity. Some sites registered up to 59 ppm (41 mg/m^3), which is above the levels recommended for humans (NOHSC 1995).

Dry and wet bulb temperatures (T_{DB}, T_{WB}) and dew point (DP) are strongly positively correlated with NH_3 accumulation (Pines & Phillips, 2011), and these could provide auxiliary information for NH_3 monitoring. High temperatures during export from Australia to the Middle East could cause the reduction of feed intake in sheep (Stockman, *et al.* 2011), in the same way as high NH_3. The two are inter-related. Combined with insufficient ventilation, high temperatures and relative humidity (RH) contribute to NH_3 volatilisation, and exposure to a high NH_3 environment could reduce animals' evaporative heat loss via increased respiratory rate, because of detrimental effects on normal respiratory function (Costa, *et al.* 2003). Panting becomes common in sheep when maximum daytime T_{DB} reaches 26^{o}C, which is normally exceeded by the end of the voyage during live export from an Australian winter to a Middle East summer (Thwaites 1985; Phillips 2016). However, only one average recording for T_{DB}, T_{WB} and humidity per deck each day is required by the Australian Standards for the Export of Livestock (DAFF 2011), which is usually recorded in the morning, in order to report this to the captain later in the day. This is probably not indicative of the maximum daytime temperature. The location for temperature measurement is not prescribed and could be in the

best ventilated part of each deck. Moreover, temperatures might be under-reported by ship veterinarians, who are employed by the livestock exporting companies (Caulfield, *et al.* 2014).

The Australian live export industry has developed a Heat Stress Risk Assessment model (Ferguson, *et al.* 2008; Caulfield, *et al.* 2014). Although published details are scant, the model uses predicted T_{WB} en route and at the destination port, as well as ship factors, such as ventilation rate. However, it has been criticised for, amongst other things, not being validated against performance data (Ferguson, *et al.* 2008; Caulfield, *et al.* 2014) and ignoring important ship characteristics, such as the difference between open and closed decks (McCarthy 2005). For effective validation against performance, it is important to know the temperature on board accurately, but there is currently no validated sampling strategy to achieve this.

Ammonia concentration varies spatially on live export vessels due to ventilation rates, temperatures and the extent of faecal accumulation on the decks (Pines & Phillips, 2011). High NH_3 in one shipboard study occurred particularly on closed decks, as well as at the front of the vessel and near the engine block on open decks (Pines & Phillips, 2011). The number of sampling sites determines the spatial resolution of the NH_3 concentration profile, but this is often constrained by equipment, time, and manpower. The growing use of electronic measurement systems may make it possible to sample automatically on ships, as has been done in livestock buildings by drawing air from multiple sites to a central electronic gas measurement unit (Groot Koerkamp, *et al.* 1998; Phillips, *et al.* 1998a; Banhazi 2009). Because the labour input associated with routine measurements is one of the major costs of monitoring, the use of electronic monitoring systems could help reduce the cost required for operating the system and processing the data (Banhazi 2009). However, it must be noted that the environment on livestock shipment is hostile for electronic measurement, damp and without electricity. The sampling devices must be out of reach of the animals, but NH_3 concentration decreased with height above the deck in one study, on average from about 19 ppm (13 mg/m^3) at sheep height to about 17 ppm (12 mg/m^3) at a height safe from damage by sheep (Pines & Phillips 2011). However, no matter what monitoring method is used, selection of the most representative sampling sites on the shipment is of practical value for NH_3 monitoring (Ni & Heber 2008), and probably for T_{DB} and RH measurement.

Because of the random and systematic errors from sampling and analytical operations, obtaining the true value of an environmental parameter is an idealisation. The closeness of an individual measurement value or the average of multiple measurements to the true value reflects the effectiveness and accuracy of measurement (USEPA 2002). Comparison of measured values with the

true value, obtained using a 'gold standard' measurement technique, has been an approach adopted in previous studies investigating NH_3 in different livestock buildings (Groot Koerkamp, *et al.* 1998; Hinz & Linke 1998; Parbst, *et al.* 2000; Phillips, *et al.* 1998a).

Precision determined by the statistical distribution of random errors is used as an uncertainty value to measure agreement among replicated measurements of the same object, under similar conditions. Bias is related to systematic error caused by the systematic or persistent distortion involved in a measurement process (USEPA 2002). Uncertainty of measurement reflects the dispersion of the values, which could be attributed to the quantity measured. Therefore, in order to obtain an optimal measurement protocol or reduce measurement efforts, uncertainty analysis can be used to evaluate the measurement precision by giving a quantitative estimation of confidence in the data (Sharp 1999).

This study aimed to develop a sampling strategy for the measurement of micro-climatic conditions during live export based on an analysis of measurement uncertainty. Due to the contentious nature of the trade, access to live export shipments by scientists is rarely allowed, therefore a database obtained from two shipments of live sheep from Australia to the Middle East was utilised. Developing a sampling strategy for NH_3, T_{DB} and RH measurement is essential to interpret livestock mortality and animal welfare risk management during live export (Phillips 2016).

4.3 Material and methods

4.3.1 Datasets

Voyages and measurements

Two datasets were used to estimate the numbers of sampling sites required for NH_3, T_{DB} and RH measurement on live export vessels from Australia to the Middle East. The datasets originated from measurements on two voyages undertaken by a vessel regularly used for live sheep export in Australia (Pines & Phillips 2011). Shipments transporting sheep from Australia to the Middle East usually carry approximately 75 000 sheep in vessels with about nine decks. Decks close to or below the water line are fully enclosed to prevent seawater incursion and rely on forced ventilation. Higher decks are open-sided, using both forced and natural ventilation to cool the livestock, with natural ventilation being dependant on orientation of the ship and wind speed. In Pines & Phillips' research, environmental conditions were studied on a 177 × 31 m vessel of 10 973 t, which carried approximately 70 000 sheep in 779 pens installed on five closed decks and four open decks. Closed decks were mechanically ventilated by 84 reversible blowers operating at 60 air changes per hour, according to the ship specifications (Pines & Phillips 2011).

The T_{DB}, DP and air speed were measured at standing sheep head height (approximately 60 cm above the pen floor) on both voyages (Pines & Phillips, 2011). The RH was estimated from T_{DB} and DP by an RH Calculator (Bureau of Meteorology, Australian Government).

On the first voyage, twenty measurement sites were selected for collection of all environmental data: ten predicted High NH_3 sites and ten predicted Low NH_3 sites. High NH_3 sites were chosen based on previous research investigating NH_3 levels on this vessel (Accloly, J., personal communication) and communication with the ship boatswain. On the same decks ten Low NH_3 sites were selected in areas with high ventilation rates and pens where air flow was not disrupted. High and Low NH_3 sites were paired on the basis of class of sheep, pen size and stocking density (Table 4-1, from Pines & Phillips 2011), with 4 pairs located on closed decks and 6 pairs on open decks. Data was collected over 11 days, with each individual site visited at approximately the same time between 09:00–16:00 h on each day to avoid influence of circadian fluctuation in concentrations and temperature (Pines & Phillips 2011). Data obtained on this voyage were used to estimate the numbers of sampling sites required for NH_3, T_{DB} and RH measurement on the entire vessel, as well as evaluating this separately on decks that were open to the atmosphere and those that were closed.

On the second voyage, data collection occurred in eighteen sheep pens, which, based on the results of the first voyage, were evenly divided between nine Low and nine High NH_3 levels. Within each pen, data were collected at sixteen sampling sites selected every 2.2 m in both a port/starboard and fore/aft directions. Three pens were recorded each day over days 3 to 9 during the second voyage (Pines & Phillips 2011). Data obtained on this voyage were used to estimate the numbers of sampling pens and sampling sites within sheep pens required for NH_3, T_{DB} and RH measurement on the vessel.

Measuring devices

On the first voyage, NH_3 was measured using a Neotox Mk Ammonia Meter (Nutech Australia, Western Australia, Australia) (accuracy: ± 5 ppm (3.5 mg/m^3); resolution: 1 ppm (0.7 mg/m^3)) and Gastec gas detection tubes (Gastec Corporation, Fukaya, Japan) (coefficient of variation 5–10%; resolution: 1 ppm (0.7 mg/m^3)). Prior to the voyages the Neotox meter was calibrated and fresh air calibration was conducted before each measurement. Readings from Gastec tubes and the Neotox meter were highly correlated (correlation coefficient = 0.94, $P < 0.001$), especially below 20 ppm (14 mg/m^3), where most readings lay (Pines & Phillips 2011). A third set of measurements was attempted with a QRaePlus Meter but this failed during the voyage. Data collected by the gas

detection tubes were used for analysis because of apparent greater accuracy. On the second voyage, NH_3 was measured using the same Neotox meter and QRae gas detection tubes, and a QRae Plus meter (RAE Systems, California, USA) (relative standard deviation (SD) <12%, resolution: 2 ppm (1.4 mg/m^3)). Readings from both brands of gas detection tubes were corrected for T_{DB}. On the second voyage, readings from the Neotox meter were used for statistical analysis, because of insufficient QRae tubes and unstable readings from the QRae Plus meter.

A Kestrel 4000 Pocket weather station (Nielsen Kellerman Australia, ACT, Australia) was used to measure T_{DB} (accuracy: ± 2%; resolution: 0.1 ℃) and DP (accuracy: ± 2%; resolution: 0.1 ℃). In order to ensure accuracy, readings were compared regularly to those obtained from a second hygrometer (PCWI Model 8705 Hygrometer, PCWI Precision Instrumentation, NSW, Australia). Two digital anemometers were used to measure air speed on the both voyages: a CFM Master 8901 meter (accuracy: ± 2%; resolution: 0.01 m/s) and the Kestrel weather station referred to above (accuracy: ± 3%; resolution: 0.3 m/s); results are presented from the former instrument due to its greater accuracy.

4.3.2 Development of virtual sampling schemes

On the basis of data on the first voyage, different numbers of site pairs, from 2 to 10 were included to develop different sampling schemes for estimation of sampling sites required for NH_3, T_{DB}, and RH measurement on the entire vessel. Following a similar approach, sampling schemes on open or closed decks were evaluated, with the number of site pairs included either between 2 and 6 (open decks) or between 2 and 4 (closed decks). All possible sampling schemes were generated using Visual Basic for Applications macro in Microsoft Excel Version 2013.

Based on the data collected on the second voyage, different numbers of High or Low NH_3 pens, from 2 to 9, were included to develop different sampling schemes for estimation of sampling pens required for NH_3, T_{DB}, and RH measurement. All possible sampling schemes were created in Minitab (Version 16; Minitab Inc, State College PA, USA), with High and Low NH_3 pens evenly selected for each scheme. To determine the sampling density in each pen, different numbers of sites between 2 and 16 were randomly included to develop different sampling schemes, using a publicly available random number generator (Random.org, Randomness and Integrity Services Limited, Dublin, Ireland).

For the virtual sampling schemes with equal number of sampling sites or pens, if the size of all possible sub-datasets was smaller than 200, all sampling schemes were statistically analysed. Otherwise, 200 randomly selected sub-datasets were analysed, yielding a plot of the measurement uncertainty against the number of sampling sites or pens.

4.3.3 Statistical analysis

Statistical analysis was carried out in Minitab 16.0. Data from each virtual sampling scheme were subjected to the General Linear Model Procedure (GLM) for unbalanced analysis of variance (ANOVA), except sampling schemes between sheep pens, which were analysed by two-sample t-tests. In order to achieve normal distribution of the residuals for the GLM analysis, NH_3 data were transformed to $\log_{10}$ values and RH data transformed to squared values. The residuals of untransformed T_{DB} data analysis on closed decks of the first voyage were approximately normally distributed, as well as those of T_{DB} and square transformed RH data analysis on the second voyage.

On the first voyage, the linear components of the model contained fixed terms ("NH_3 level (High/Low)" and date), a random factor (pair) and the interaction between "NH_3 level (High/Low)" and pair as equation (1):

$$\mu + a + b + c + a.c \qquad (1)$$

where a = factor (NH_3 level (High/Low)), b = factor (date), c = random factor (pair).

On the second voyage, the linear components of the model contained fixed terms ("NH_3 level (High/Low)" and "site within pen") and a random factor ("pen" nested within "NH_3 level (High/Low)") as equation (2):

$$\mu + d + e + f \qquad (2)$$

where d = factor (NH_3 level (High/Low)), e = factor (site within pen), f = random factor ("pen" nested within "NH_3 level (High/Low)"). The $\log_{10}$ NH_3, T_{DB}, and RH^2 values were used as the response variables for data modelling on both voyages.

Due to some missing data, least squares means were used. The differences between means of High and Low NH_3 sites or pens were assessed by Tukey's test at 5% probability level. Analysis of measurement uncertainty, expressed as the margin of error (MoE) or confidence interval (CI), was used to determine the precision of different virtual sampling schemes. Based on the results of GLM analysis, a.c and f were used as the variance sources with the degree of freedom (df) as number of pairs or pens sampled minus 1. For NH_3 measurement, the standard error of the difference between

means (SED) of High and Low NH_3 sites or pens was calculated from the adjusted ("type III") sum of squares and number of sites or pens with different NH_3 level (High/Low). The MoE for the difference was determined by the SED and a critical t value for a two-tailed t-test at 95% confidence level with relevant df. The probability value (*P*) of the t-test for difference was computed based on relevant df and t-statistic value for difference.

Following a similar approach, for T_{DB} and RH measurement, the standard error of the mean (SEM) was obtained from the adjusted ("type III") mean square and total sample size of sites or pens. The MoE for means was computed as a function of SEM and a critical t value for a two-tailed t-test at 95% confidence level with relevant df.

4.4 Results

4.4.1 Ammonia sampling schemes on entire vessel or separate open and closed decks

On the first voyage, mean NH_3 concentration at the High sites was 22.16 ± 14.45 ppm (15.44 ± 10.07 mg/m^3), while mean NH_3 concentration at the Low sites was 12.62 ± 7.92 ppm (8.79 ± 5.52 mg/m^3) (Table 4-2). In order to detect a difference in NH_3 concentration between High and Low NH_3 sites on the entire shipment, at 0.05 probability level, a minimum of ten sampling sites (five predicted High and five predicted Low) were required for reliable estimation, with an average CI for the difference ranging from 1.23 to 2.35 ppm (0.86 to 1.64 mg/m^3). Sampling at less than 10 sites resulted in an increase in the average and range of MoE, and a decrease of measurement reliability (Figure 4-1). The risk factor expressed as the percentage of measurements failing to detect the difference statistically increased from 0.79% to 33% (8 sites sampling), or more for fewer than 8 sites sampled.

Similar outcomes were found for NH_3 measurement on open decks. To detect a difference in NH_3 concentrations between High and Low NH_3 sites statistically at $P < 0.05$, a minimum of 6 sites were required, with average CI for the difference ranging from 1.30 to 2.45 ppm (0.91 to 1.71 mg/m^3). The risk factor increased to 73.3% if only 4 sites were sampled (Figure 4-2).

On closed decks, three sampling schemes with the number of sites sampled between 4 and 8 all failed to detect the difference of NH_3 concentrations between High and Low NH_3 sites at 0.05 probability level, although the *P* value of 8 sites sampling (four predicted High and four predicted Low) was 0.1, with average CI of the difference from 0.84 to 3.02 ppm (0.59 to 2.1 mg/m^3) (Figure 4-3).

4.4.2 Temperature and relative humidity sampling schemes on the entire vessel or separate open and closed decks

For T_{DB} and RH measurement, the percentage measurement error in relation to the mean of each sampling scheme was used for evaluation, and a small (1%) to moderate percentage error (5%) was considered acceptable. On the entire shipment, a minimum of 6 sites (three predicted High and three predicted Low NH_3 sites) were required for reliable estimation of T_{DB}, with average MoE for means of 0.46 ℃, giving 1.4 % average error in relation to the mean T_{DB} (Figure 4-4). However, to obtain an estimation with a relative error <1%, 8 sites (four predicted High and four predicted Low NH_3 sites) were required, in which case the error decreased to 0.94%. Similar outcomes were found for T_{DB} measurement on both open and closed decks (Figure 4-5 and Figure 4-6, respectively). In order to reduce the T_{DB} measurement error to less than 5%, at least 6 sites (three predicted High and three predicted Low NH_3 sites) on open decks were required with average MoE for means of 0.57 ℃, accounting for 1.83% of the mean T_{DB}. On closed decks, a minimum of 4 sites (two predicted High and two predicted Low NH_3 sites) were required for an average MoE for means of 1.00 ℃, making up 3.09% of the mean T_{DB}. If more sites were sampled, the precision was improved, with percentage measurement error less than 1%. Sampling at 10 sites on open decks or 6 sites on closed decks was necessary to reduce the error further to 0.93% or 0.78%, respectively (Figure 4-5).

Compared with T_{DB} data, RH data were more variable. With the number of sites sampled increased, the precision was only improved slightly. On the shipment, even sampling at maximum 20 sites (ten predicted High and ten predicted Low NH_3 sites), the average MoE for means was 10.93%, accounting for 14.80% of the mean RH (Figure 4-4). Based on an exponential model describing the decay of MoE against the number of sampling sites, the minimum measurement error that could be achieved was 13.82% (Figure 4-7). Following a similar approach, even sampling at the maximum 12 sites on open decks or 8 sites on closed decks, the average MoE for means was 12.25% or 17.47%, accounting for 16.40% or 24.07% of the mean RH (Figure 4-6). The minimum measurement errors on open or closed decks that could be achieved were 13.20% and 19.67%, respectively, based on exponential models describing the decay of MoE against the number of sampling sites.

4.4.3 Ammonia sampling schemes between or within sheep pens

On the second voyage, mean NH_3 concentration in High NH_3 pens was 22.91 ± 7.98 ppm (15.96 ± 5.56 mg/m^3), while mean NH_3 concentration in Low NH_3 pens was 13.97 ± 2.72 ppm (9.73 ± 1.89

mg/m^3) (Table 4-2). To detect the difference between High and Low NH_3 pens statistically at a 0.05 probability level, a minimum of 10 pens (five predicted High and five predicted Low) were required with an average CI for the difference of 1.16 to 2.21 ppm (0.81 to 1.54 mg/m^3) (Figure 4-8). Sampling in 8 pens increases the average MoE, and the risk factor, indicating the percentage of measurements failing to detect the difference statistically, increased to 19%. In contrast, within each pen, only 2 sites were necessary to detect differences between High and Low NH_3 pens, with average CI for the difference from 1.24 to 2.04 ppm (0.86 to 1.42 mg/m^3) (Figure 4-9).

4.4.4 Temperature and relative humidity sampling schemes between or within sheep pens

For T_{DB} measurement, as the number of sheep pens sampled decreased, the average MoE for means and the range exhibited a gradual increase (Figure 4-10). To obtain a relative measurement error less than 5%, ten sheep pens (five predicted High and five predicted Low) were required to be sampled, with average MoE for means of 1.28 ℃, accounting for 4.50% of the mean T_{DB}. Within each pen, two sampling sites were sufficient to obtain a percentage measurement error of 3.11%, which is less than 5% of the mean T_{DB}, with average MoE for means of 0.88 ℃ (Figure 4-10).

For RH measurement, consistent with our previous results, even sampling in the maximum 18 pens or at 16 sites within each pen, the average MoE for means was 19.9%, accounting for 26.3% of the mean RH (Figure 4-11). According to the exponential model describing the decay of MoE against the number of sampling sites, the minimum percentage measurement errors between or within pens that could be achieved were 21.2% or 26.3%, respectively.

4.5 Discussion

Ammonia and environmental measurement variations are a function of the variation between sites and the measurement accuracy. The deck maps for NH_3 (Figure 4-12, from Pines & Phillips 2011) indicated major variation between sites and clustering of High and Low NH_3 readings.

High NH_3 concentrations occurred particularly on closed decks, at the front of the shipment and near the engine block on open decks, because of a strong negative correlation between NH_3 concentration and air flow (Pines & Phillips 2011). This is why three different sampling schemes on closed decks all failed to detect the difference between High and Low NH_3 sites at a 0.05 probability level, in contrast to the number of sampling sites required for NH_3 measurement on open decks (Figure 4-3). Due to the dependence on ventilation and the potential unevenness of air flow within the confines of the ship, NH_3 concentrations on closed decks were generally higher and more variable than on open

decks (Pines & Phillips 2011), as reflected by the high SD of NH_3 measurements (Table 4-2). Therefore, caution should be used when estimating the number of sampling sites for NH_3 measurement on closed decks.

Ammonia measurement between sheep pens requires more sampling units compared to sampling within pens. This is explained by the fact that air speed varied considerably in most pens on open decks, with little or no air flow detected in 65% of the pens, although ventilation shafts were evenly distributed throughout the vessel (Pines & Phillips 2013). Variation in ventilation rates around the vessel is influenced by the proximity to the engine block, the efficiency of natural and mechanical ventilation, and the type of animals (Phillips 2008). Open decks may be subject to greater variation over time, due to varying ambient wind speeds, particularly in the outer pens exposed to prevailing winds. In port, wind speeds are likely to be much reduced due to the structural impediments to wind flow, compared with when the vessel is at sea. Theoretically, the captain may change the course of the ship's direction to increase ventilation, however, in the Arabian Gulf or similar narrow channels this is not possible.

Sheep normally are confronted with high air temperatures and humidity when shipments travel through tropical and equatorial regions during live export from Australia to the Middle East (Pines & Phillips 2011). A composite index of T_{DB} and RH, the temperature–humidity index (THI), has been proposed as a mean to estimate the severity of heat stress (LPHSI 1990; Marai, *et al.* 2007). The equation (3) to calculate THI for sheep is as follows:

$$THI = T_{DB} - \{(0.31 - 0.31\ (RH)/100)\ (T_{DB} - 14.4)\} \quad (3)$$

Where RH is measured in % and T_{DB} is measured in ^{o}C. According to Marai, *et al.* (2007), a THI value below 22.2 suggests absence of heat stress; between 22.2 and 23.3 signifies moderate heat stress; between 23.3 and 25.6 indicates severe heat stress; and > 25.6 implies extreme severe heat stress. A T_{DB} of 26 ^{o}C is normally exceeded by the end of the voyage during live export from an Australian winter to a Middle East summer (Phillips 2016). The RH levels commonly reached 85% for sustained periods during the voyages (MLA 2001). We have calculated that, from Marai, *et al.*'s (2007) equation, T_{DB} levels corresponding to critical THI levels at RH levels of 85% or 75% are: Level 1 (THI = 22.2), 22.58 and 22.86 ^{o}C, respectively; Level 2 (THI = 23.3), 23.73 and 24.05 ^{o}C, respectively; Level 3 (THI = 25.6), 26.15 and 26.54 ^{o}C, respectively. The RH levels corresponding to critical THI levels at T_{DB} levels of 26 or 27 ^{o}C are: Level 2 (THI = 23.3), 25 and 5%, respectively; Level 3 (THI = 25.6), 89 and 64%, respectively.

In contrast to NH_3 measurement, data from T_{DB} measurement on open decks were more variable than those on closed decks, with greater average MoE for means generated from sampling schemes with same number of sites selected. This may explain why a previous shipboard study found no correlation between T_{DB} and NH_3 concentration on open decks (Pines & Phillips 2011).

Besides large amount of heat generated by livestock within a ship, high temperature generally occurred in some sections of the ship, such as beside an engine room, boiler room or heated fuel tank. The Australian Government regulations require that any temperature increase in adjacent livestock space is less than 3 ^oC, when ambient temperature exceeds 22 ^oC (Australian Government 2006). In addition, the analysis of long-term meteorological datasets and regional climate model research for the 21st century suggest that the Middle East is likely to be greatly affected by climate change with increases in drought intensity and frequency and hot weather conditions (Lelieveld, *et al.* 2012). The extremes of T_{WB} in the Arabian Gulf are likely to be above 35 ^oC predicted in a regional climate change model (Pal & Eltahir 2016), making on-board monitoring even more important. Additionally, the annual Eidh al-Addha festival, which is responsible for most of the sheep exported to Saudi Arabia advances by 10 days each year. In 2016, the festival was held in mid-September, so over the next decade, sheep exported by sea from Australia to the Middle East for the purpose of sacrifice at the festival will experience high summer when temperatures are at their peak.

High RH during live export is particularly associated with high stocking density, inadequate ventilation, or the ingress of sea water on lower decks, depending on the vessel structure design and vigilance of the master and crew (Phillips 2008). An analysis of ship Master's reports demonstrated that RH commonly measured on the ship's bridge on live export vessels was in the range 70-90% (Norris & Richards 1989). In this study mean RH level on the entire shipment (voyage 1) or in the sheep pens (voyage 2) was 75% with SD of 11% and 5%, respectively. Compared to the measurement on the entire vessel, RH levels were less variable in sheep pens with lower SD (Table 4-2). Hence it seems likely that large amounts of moisture in sheep pens, which are generated by metabolic processes and released from livestock kept at high stocking density within a ship, cannot be dissipated by on board ventilation (Caulfield, *et al.* 2014). It is possible that the variation in ventilation rate within a pen, as a result of different proximity to the outlet port, caused the high variability in RH, with air-borne moisture equilibrating more slowly than temperature, which equilibrates rapidly by convection. Accuracy of DP and T_{DB} measuring devices were similar, so this is unlikely to be the reason. The high RH in the pens explains why T_{WB} (an environmental measure taking humidity into account; Hemp 1989) is normally several degrees higher in the pens than that

on the bridge (Maunsell Australia 2003). Furthermore, T_{WB} in the air leaving a pen area can vary considerably and is up to 4 ℃ higher than air entering the pen (McCarthy 2005).

As the selection of initial sampling sites on the two voyages was according to the predicted NH_3 concentration, the outcomes for T_{DB} and RH measurement in this study should be further tested with site selection based on T_{DB} or RH distribution on live export vessels.

Variations in NH_3 concentration and temperature over time are also important in that subjects may attenuate their responses or become hypersensitive in response to integrated concentrations. On the first voyage NH_3 concentration gradually increased over time on the closed decks, whereas on the open decks it was variable because of changing natural ventilation rates (Pines & Phillips 2011). There is little definite evidence of changing responsiveness over time in animals; in the only relevant study with sheep exposed to NH_3, repeated exposure did not change the responses (Phillips, *et al.* 2012a), and Jones, *et al.* (2005) found no effect of early exposure for 26 days on the avoidance of NH_3 by chickens. However, some studies suggest that humans can acclimatize to NH_3, with a gradual reduction in responsiveness over time (Ferguson, *et al.* 1977; Harada, *et al.* 1983; Schiffman 1998). Despite this, Holness, *et al.* (1989) reported no change in odour sensitivity in humans chronically exposed to moderate levels of NH_3 (12.5 ppm, 8.71 mg/m^3). In relation to temperature, repeated exposure of livestock to high temperatures under simulated shipboard conditions brings increased responses (Stockman, *et al.* 2011; Caulfield, *et al.* 2014).

There are several limitations to this study. Firstly, accuracy of the measuring devices is a limitation. The values we quoted are from manufacturers' statistics, which may not be accurate. Other authors have quoted great variation in the levels of imprecision measured for gas tubes, e.g. SD from 7 to 20% (Ni & Heber 2008). Our study used data from two measuring devices, and the relationship between them was determined at the time of measurement (Pines & Phillips 2011). Secondly, in our study the measurements were taken at similar times on each day, therefore there is also a limitation that circadian fluctuation in measurements was not taken into account. Thirdly, all of the virtual sampling strategies evaluated in this study were based on just two live sheep export voyages of a single ship travelling from Australia to the Middle East. The results and conclusions should be further tested and verified with data from other sheep shipments, especially for NH_3 measurement on closed decks and RH measurement. In addition, measurements of NH_3, T_{DB} and RH on actual voyages of live sheep export from Australia to the Middle East would be required to validate the virtual sampling strategies obtained from this study. Our results may be valid for other ships with similar characteristics being used for long distance transport of sheep, but there are also many

smaller shipments over shorter distances that would need to be separately evaluated. Our methodological approach could be used to calibrate other vessels, prior to establishment of sampling protocols for micro-climatic condition measurement within livestock vessels.

4.6 Conclusions

This study suggests that measurements of ammonia and dry bulb temperature on the shipment of live sheep export from Australia to the Middle East appeared achievable, with approximately ten and six to eight sampling sites required, respectively. However, relative humidity, an important contributor to the stress caused by both temperature and ammonia, could not be reliably measured with even twenty sampling sites. The number of pens measured contributed more to the variance of ammonia and dry bulb temperature measurement than the number of sampling sites within each pen on open decks.

When estimating the number of sampling sites required for effective environmental monitoring on live export vessels, not only the measurement reliability but also the cost and time input required to be taken into account. This increases with the number of sampling sites, therefore the investment in time and money should be balanced against the desired measurement precision required for animal welfare assessment. An understanding of effective sampling strategies, including those with electronic monitoring, will enable livestock exporters to improve animal welfare during live export by efficiently monitoring the micro-climatic conditions and taking appropriate actions to control excesses.

4.7 Tables and figures

Table 4-1. Description of study sites on voyage one (from Pines & Phillips, 2011)

Site	Predicted NH_3	Deck (top-bottom	Deck type (Open/ Closed)	Starboard, (1)- Port (4)	Fore/Middle/Aft	Nearest vent. unit (m)	Sheep type	Stocking density (m^2/head)	Pen area (m^2)
1a	High	9	Open	4	Fore	12	55 kg wether	0.351	71
1b	Low	9	Open	4	Middle	4	55 kg wether	0.351	74
2a	High	9	Open	3	Fore	12	40 kg lamb	0.290	71
2b	Low	9	Open	3	Middle	4	40 kg lamb	0.290	70
3	High	8	Open	2	Fore	12	55 kg wether	0.351	71
3	Low	8	Open	2	Middle	4	55 kg wether	0.351	71
4	High	8	Open	4	Middle	9	53 kg ewe	0.337	71
4	Low	8	Open	2	Aft	2	53 kg ewe	0.337	70
5	High	7	Open	3	Fore	9	60 kg wether	0.425	89
5	Low	7	Open	3	Middle	4	60 kg wether	0.425	71
6	High	6	Open	1	Middle	7	55 kg wether	0.351	121
6	Low	6	Open	2	Middle	4	55 kg wether	0.351	95
7	High	2	Closed	3	Aft	6	46 kg wether	0.305	106
7	Low	2	Closed	4-5	Aft	4	46 kg wether	0.305	167
8	High	1	Closed	1-2	Aft	4	46 kg wether	0.305	106
8	Low	1	Closed	1-2	Aft	2	46 kg wether	0.305	106
9	High	2	Closed	3	Fore	4	46 kg wether	0.305	84
9	Low	2	Closed	4	Fore	4	46 kg wether	0.305	89
10	High	1	Closed	2	Fore	9	46 kg wether	0.305	78
10	Low	1	Closed	3	Fore	4	46 kg wether	0.305	89

Table 4-2. Basic statistics for ammonia (NH_3), dry bulb temperature (T_{DB}), and relative humidity (RH) measured in two voyages

	N	Mean	SD	Minimum	Median	Maximum
Voyage1						
NH_3 at high NH_3 sites (ppm)	99	22.16	14.45	1.80	15.30	58.81
NH_3 at low NH_3 sites (ppm)	100	12.62	7.92	2.25	11.64	43.47
NH_3 on the entire ship (ppm)	199	17.36	12.55	1.80	13.35	58.81
NH_3 on open decks (ppm)	115	11.49	7.47	1.80	10.56	44.46
NH_3 on closed decks (ppm)	84	25.40	13.64	8.90	20.31	58.81
T_{DB} on the entire ship (℃)	199	31.42	2.63	23.50	31.40	37.40
T_{DB} on open decks (℃)	115	30.72	2.53	23.50	30.80	36.90
T_{DB} on closed decks (℃)	84	32.36	2.47	25.90	32.25	37.40
RH on the entire ship	199	0.75	0.11	0.30	0.78	0.89
RH on open decks	115	0.77	0.09	0.30	0.78	0.88
RH on closed decks	84	0.73	0.13	0.32	0.77	0.89
Voyage2						
NH_3 in high NH_3 pens (ppm)	144	22.91	7.98	13.00	20.00	59.00
NH_3 in low NH_3 pens (ppm)	144	13.97	2.72	7.00	14.00	20.00
NH_3 (ppm)	288	18.44	7.45	7.00	17.00	59.00
T_{DB} (℃)	288	28.41	1.94	23.10	28.80	31.70
RH	288	0.75	0.05	0.64	0.76	0.86

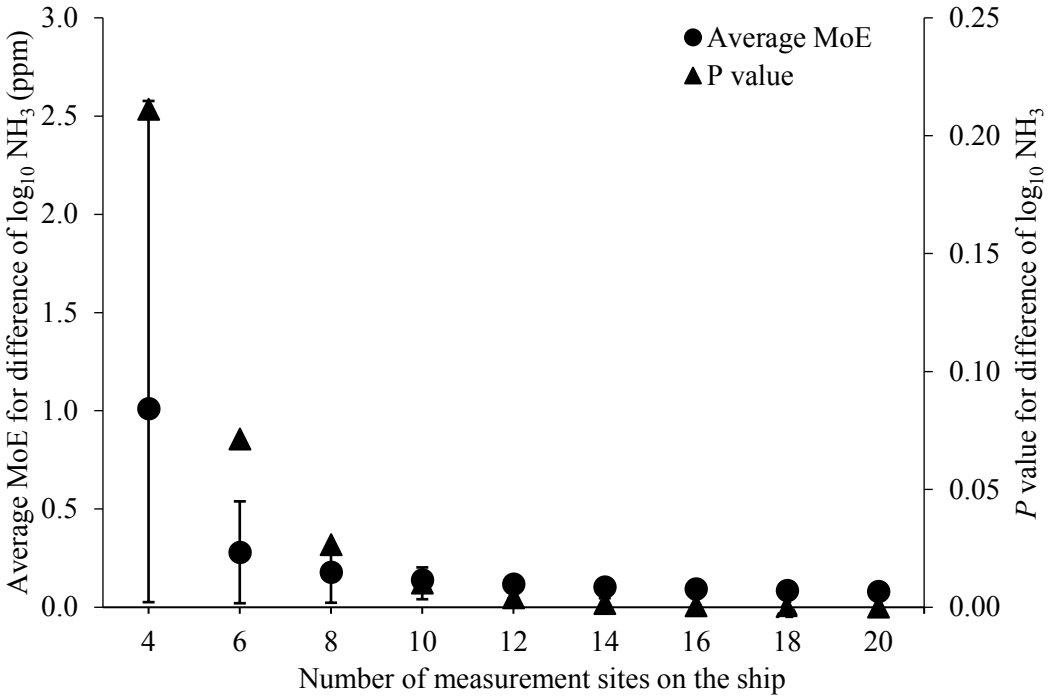

Figure 4-1. Average MoE and *P* value for difference of $\log_{10}$ NH_3 between High and Low NH_3 sites on entire vessel, with maximum and minimum MoE as error bars.

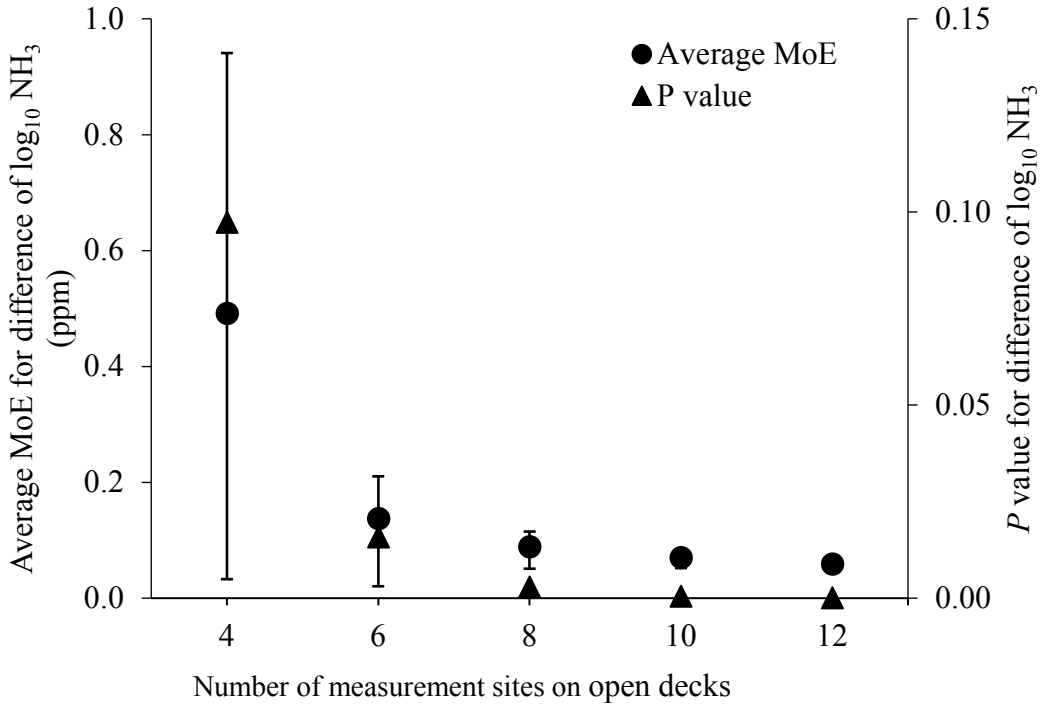

Figure 4-2. Average MoE and *P* value for difference of $\log_{10}$ NH_3 between High and Low NH_3 sites on open decks, with maximum and minimum MoE as error bars.

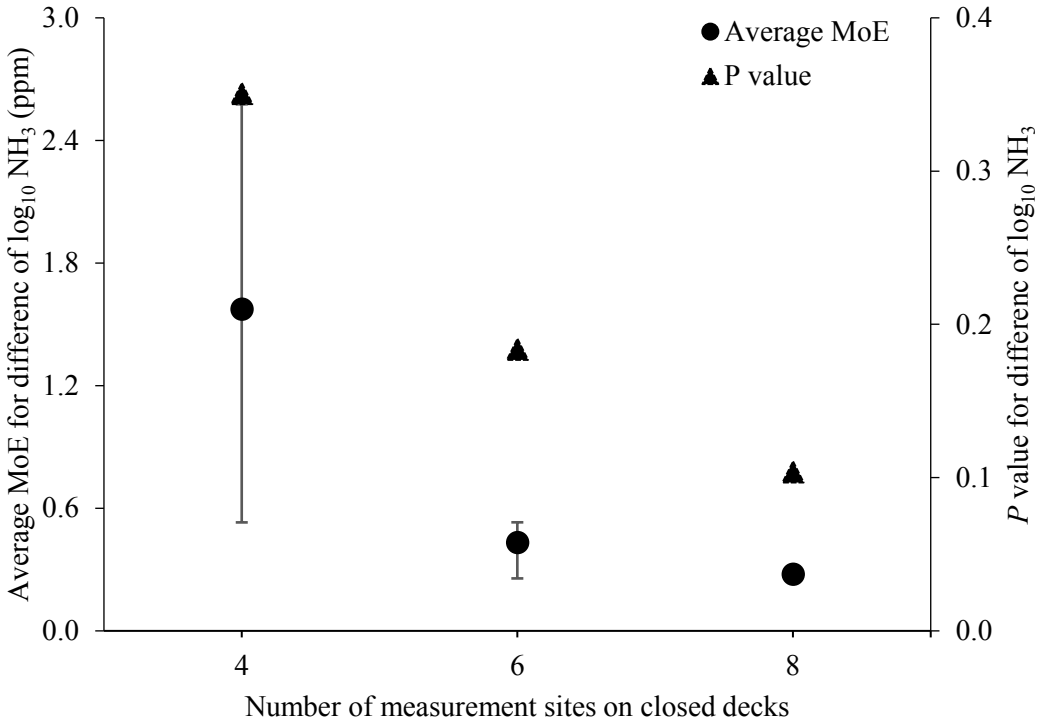

Figure 4-3. Average MoE and *P* value for difference of $\log_{10}$ NH_3 between High and Low NH_3 sites on closed decks, with maximum and minimum MoE as error bars.

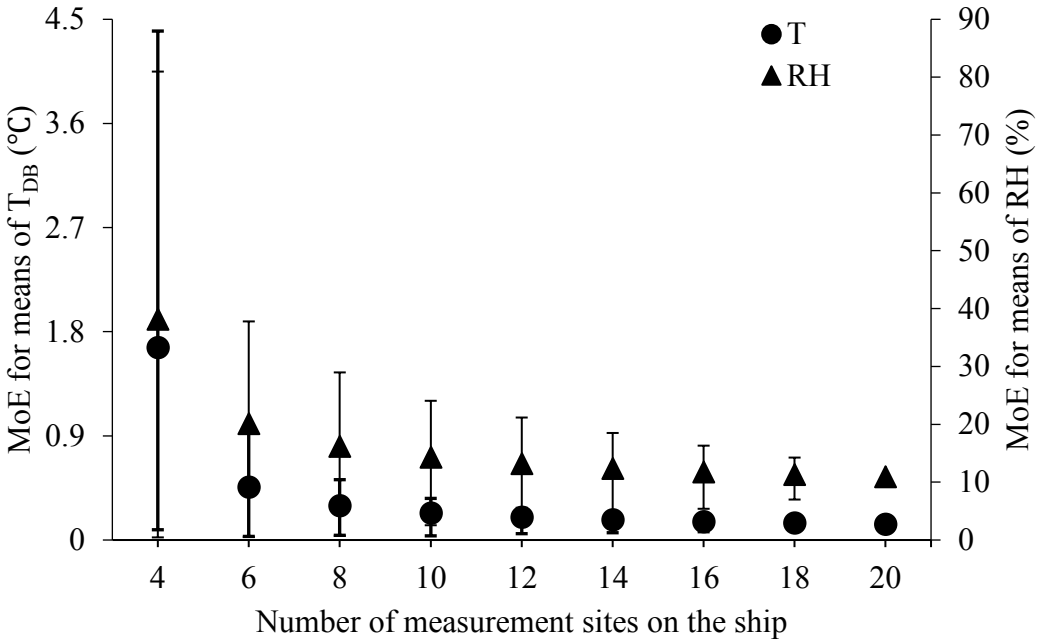

Figure 4-4. Average MoE for means of T_{DB} and RH on entire vessel, with maximum and minimum MoE as error bars.

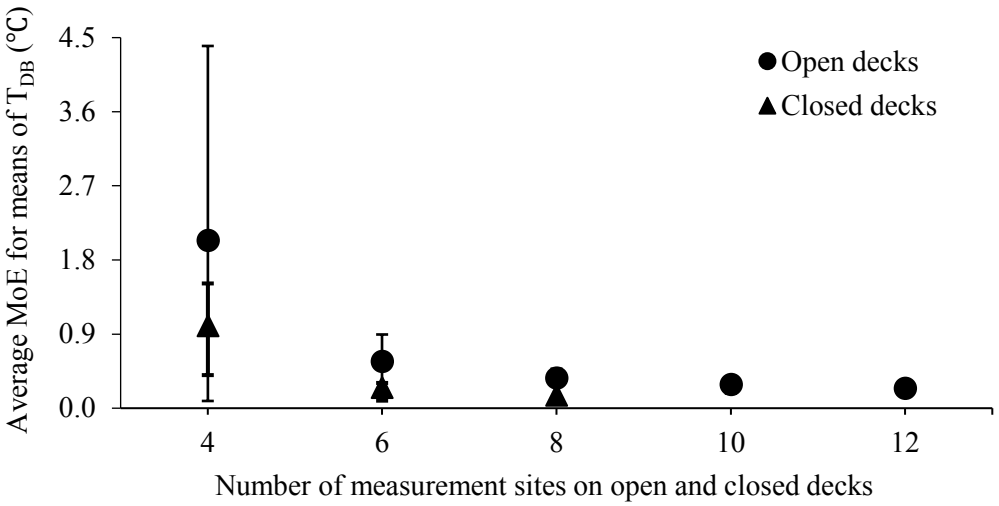

Figure 4-5. Average MoE for means of T_{DB} on open and closed decks, with maximum and minimum MoE as error bars.

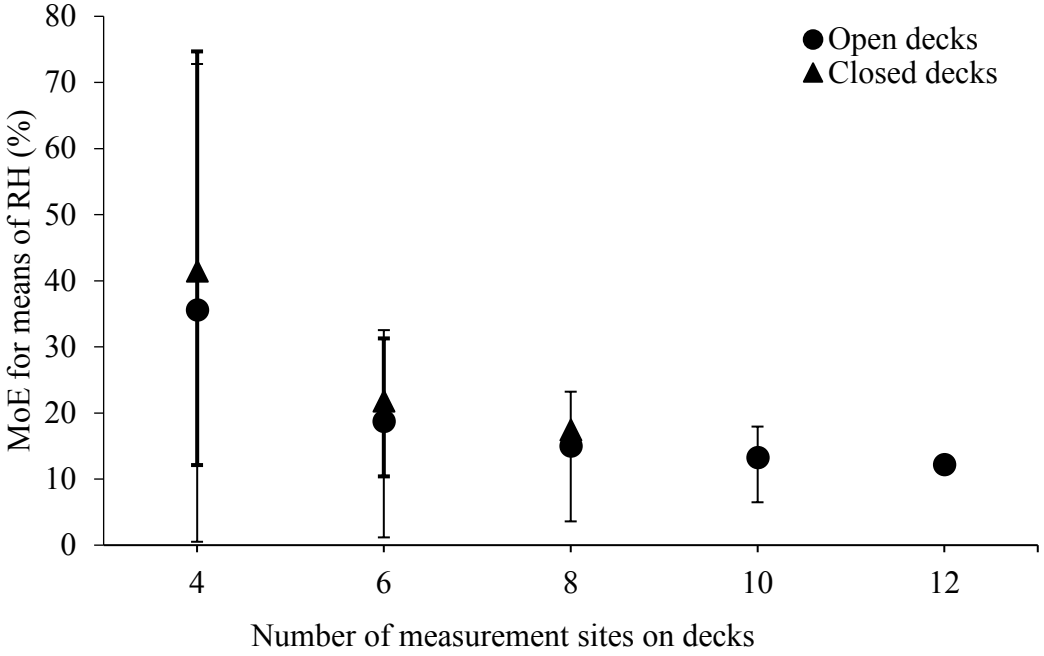

Figure 4-6. Average MoE for means of RH on open and closed decks, with maximum and minimum MoE as error bars.

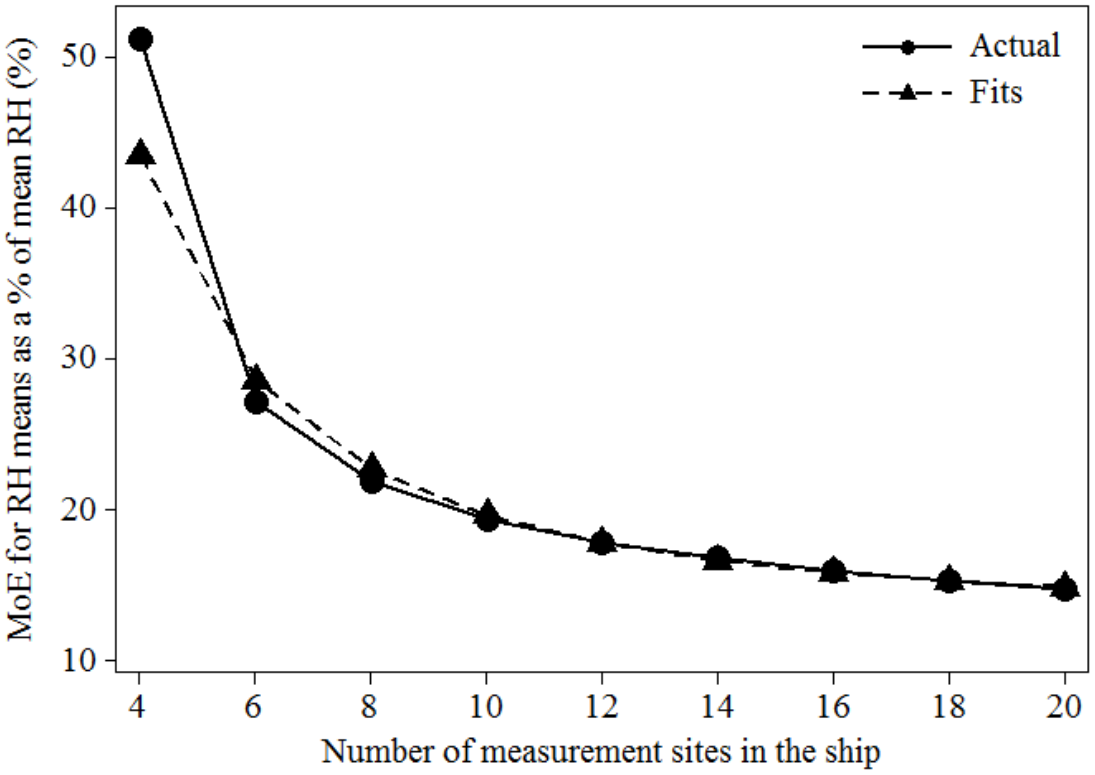

Figure 4-7. S-curve trend analysis plot for RH MoE, as a % of mean RH (%). Equation MoE = $10^3/(72.38 - 65.20\times0.76^n)$, with asymptote of 13.8; n = number of sites on the ship.

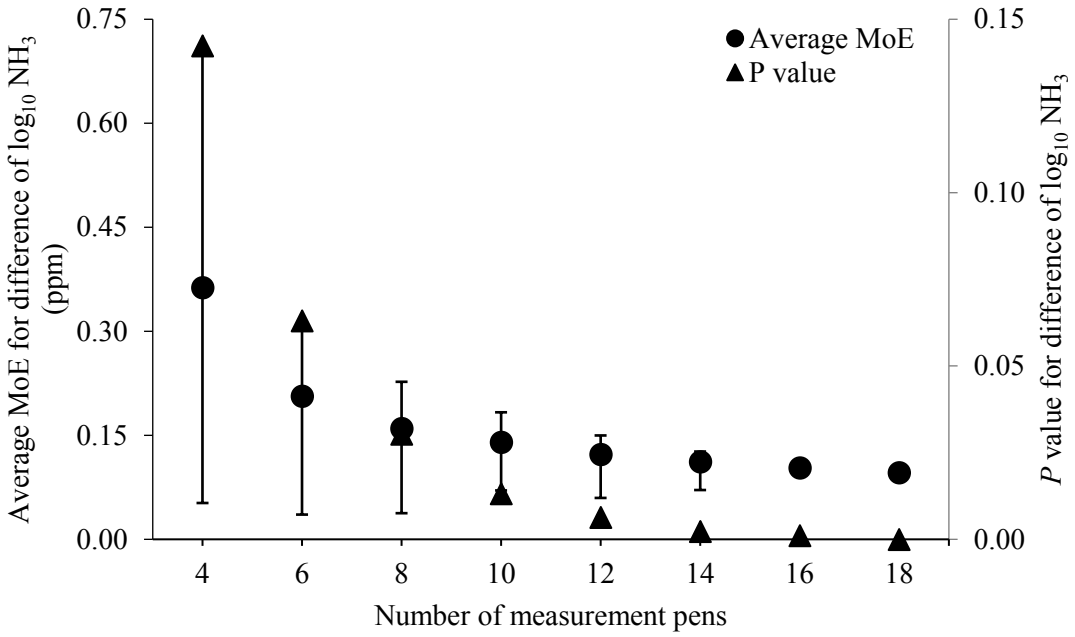

Figure 4-8. Average MoE and *P* value for difference of $\log_{10}$ NH_3 between High and Low NH_3 pens in different pen sampling schemes, with maximum and minimum MoE as error bars.

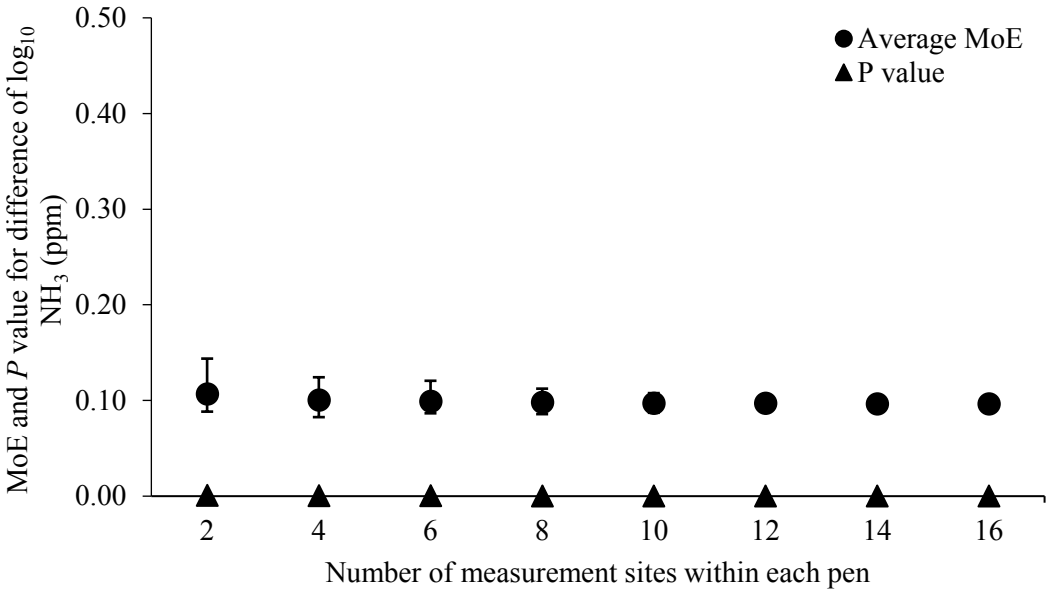

Figure 4-9. Average MoE and *P* value for difference of $\log_{10}$ NH_3 between High and Low NH_3 pens in different sampling schemes within pens, with maximum and minimum MoE as error bars.

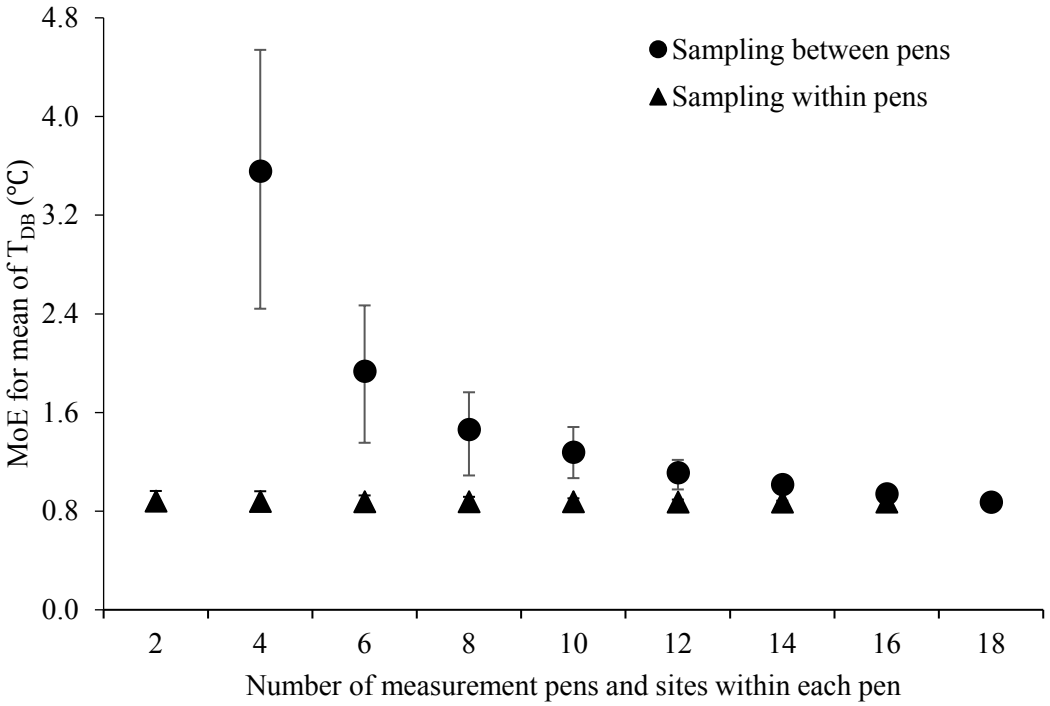

Figure 4-10. MoE for means of T_{DB} between and within High and Low NH_3 pens, with maximum and minimum MoE as error bars.

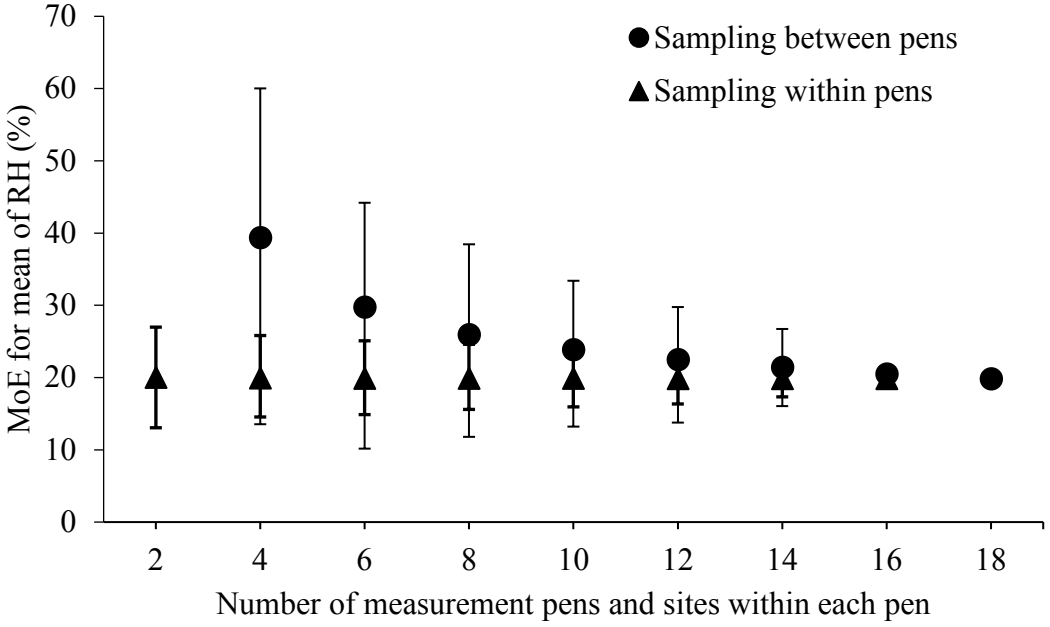

Figure 4-11. MoE for means of RH between and within High and Low NH_3 pens, with maximum and minimum MoE as error bars.

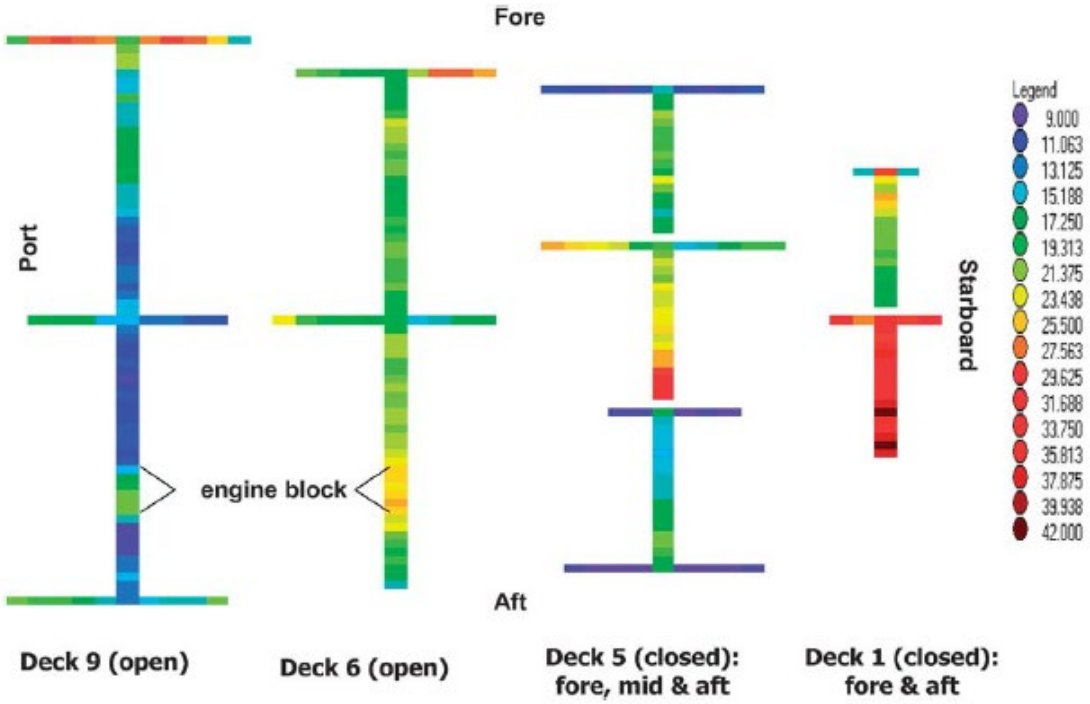

Figure 4-12. Mapping of ammonia on four decks on voyage two. Transects along the length and breadth of two open or closed decks. Deck 5 is divided into three discrete subsections and deck 1: two subsections (from Pines & Phillips, 2011).

5 Chapter 5: General discussion

Mortality is currently the most important indicator for sheep welfare assessment during live export from Australia to the Middle East. The normal causes are not well documented, but in the early years of live export 43% were attributed to inanition (Richards, *et al.* 1989). However, little is known about the contribution of the micro-climatic conditions (in particular ammonia accumulation in sheep pens) on the shipments and the climatic conditions before embarkation to the mortality of sheep during long-distance transport by sea. Thus, this project makes an important contribution to investigating the influence of these factors on sheep mortality during live export by: 1) exploring the mechanisms underlying the effects of ammonia accumulation on sheep feed intake through a multidisciplinary approach (Chapter 2); 2) exploring the seasonal mortality pattern in sheep export based on a large dataset and 3) identifying the key correlates of sheep mortality (Chapter 3). In addition to this novel evidence, which allows for the pre-emptive action be taken to reduce mortality risks in sheep export, this project also developed an effective sampling strategy for ammonia, temperature and relative humidity measurement on board shipment during live sheep export (Chapter 4).

5.1 Ammonia exposure and mortality-related feed intake reduction

A level of 30 ppm (21 mg/m^3) was used for the Ammonia treatment in this study (Chapter 2), which is a physiologically-validated maximum ammonia concentration proposed for live steer export. This study not only confirms the adverse effect of ammonia exposure on sheep feed intake, but also demonstrates lowered chewing rates during eating and rumination in sheep exposed to ammonia at a similar concentration to those experienced by sheep during export by sea. Together with supporting evidence from previous ammonia research on sheep export (Phillips, *et al.* 2012a), the measurable influence found in this study provides new evidence to consider this exposure level a suitable maximum ammonia concentration for live sheep export, which is not currently regulated by the Australian Standards for the Export of Livestock (DAFF 2011). Similarly, adverse effects of ammonia released from the manure accumulation on feed intake, body weight gain, and feed conversion efficiency have been documented in pigs and poultry (Stombaugh, *et al.* 1969; Drummond, *et al.* 1980; Yahav 2004), but little is known about the mechanisms involved. Therefore, this study suggests that similar investigation in these species should be considered.

In this study, a 50:50 mixed hay of Rhodes grass (*Chloris gayana*) and alfalfa (*Medicago sativa*) was used for daily feeding, and alfalfa pellets and sorghum chaff were used in the feed palatability

test. However, it must be emphasized that during live export a specific pelleted diet is used for sheep feeding (DAFF 2011). Therefore, reduced feed intake and lowered chewing rates during eating and rumination in ammonia exposed sheep demonstrated in this study could be compared with future on board investigations based on the specific shipboard ration. Under live export conditions, the measurable influence demonstrated by sheep exposed to 30 ppm (21 mg/m^3) for 14 days in this study could be increased, because: 1) unlike cattle export, the drier excreta in sheep pens is allowed to accumulate into a pad without removal until animals are discharged, and some very high ammonia concentrations (29 to 44 mg/m^3) have been detected in pens on previous voyages (Pines & Phillips 2013); 2) the voyage duration could be longer than the 14-day trial period in this study (Phillips 2008). Therefore, future research on board shipment is required to assess the influence of longer voyage duration and/or higher ammonia treatment on feed intake and nutritional behaviours in sheep exported from Australia to the Middle East.

The adverse effects of ammonia exposure on eating and ruminating behaviours represent the main significant results from this study, which were mainly interpreted as an indication of possible irritation in the buccal cavity of sheep. So far, little research has been conducted to investigate this influence in livestock, but in humans at least, the irritation of oral pharynx, oesophagus, and stomach has been reported after imbibing liquid ammonia (Sugawa, *et al.* 1981). Thus, the effects of ammonia exposure on the buccal cavity health and ruminal digestion could be investigated in future research.

In this study, although the elevated faecal corticosterone metabolites concentration on day 6 was not correlated to the reduced feed intake in ammonia exposed sheep, this physiological response was interpreted as an indication of stress caused by ammonia exposure. This interpretation was based upon the glucocorticoid 'independence', suggesting cortisol and corticosterone may respond differently to acute and chronic stressors (Koren, *et al.* 2012). However, it must be emphasized that more frequent sampling points throughout the voyage duration might be needed for future on board research to assess the stress (acute vs chronic) responses of sheep to ammonia accumulation during live sheep export. We further noted that, within the Ammonia treatment, sheep with high faecal corticosterone metabolites concentration on day 6 approached to the middle ambiguous location faster. The decreased decision-making time may indicate anxiety in some sheep exposed to ammonia (Burman, *et al.* 2009; Brydges, *et al.* 2012). Thus, future investigation on whether anxiety is induced is suggested. Although previous research in chicks suggested that the cognitive bias test can differentiate between anxiety disorders and depression (Salmeto, *et al.* 2011; Hymel & Sufka 2012), it is not suggested for future research, because this assessment typically requires extensive

training and is impractical in applied contexts, such as investigation on board shipments. In this study, no effect was observed in sheep's response to either partially positive ambiguous bucket location or middle ambiguous location after ammonia exposure, which might be because the double negative reinforcers during training were too strong, indicated by the failure of sheep to approach the partially negative ambiguous location. Therefore, to avoid the potential influence of training, the attentional bias test offers a faster method for assessing different levels of anxiety in sheep indicated by increased tendency to direct their attention towards threatening stimuli (Lee, *et al.* 2016).

In order to prevent residual effects on the sheep behaviour, the feed palatability test could not be conducted on the same days when general behaviour was video recorded continuously for 48 hours. In addition, due to a lack of clear video footage, prehension biting rate of sorghum chaff could not be analysed. Thus, instead of analysing sheep behaviour manually based on the videos recorded, sensor technologies could be considered for future research on board shipment based on the specific shipboard ration, such as acoustic monitoring of jaw movements (Ungar & Rutter 2006; Clapham, *et al.* 2011; Navon, *et al.* 2013; Deniz, *et al.* 2017), rumination (Harris, *et al.* 2001; Rutten, *et al.* 2017) and the use of movement/behaviour loggers (Schwartzkopf- Genswein, *et al.* 1999; Champion, *et al.* 2005; Keeling & Veissier 2005; Huhtala, *et al.* 2007; Brehme, *et al.* 2008; Eigenberg, *et al.* 2008; Meen, *et al.* 2015; Neethirajan 2017; Fogarty, *et al.* 2018; Nielsen, *et al.* 2018). The application of sensor technologies could significantly increase the efficiency of research undertaken either in research or commercial environments.

5.2 Seasonal mortality pattern in sheep export and its key correlates

This is the first study (Chapter 3) that demonstrates a seasonal mortality pattern in sheep during Australian live export based on a large dataset, with more deaths occurring on sea voyages arriving in the Middle East during the southern hemisphere summer or autumn than those in the southern hemisphere winter or spring. Heat stress and inadequate fat mobilization for energy supply when sheep are inappetant on shipments may explain this seasonality. This study makes an important contribution for improvement of live export from Australia to the Middle East. First, the results suggest that there could be review of the sheep trade from Australia in the southern hemisphere winter, which is already restricted for the export of *Bos taurus* cattle in the area of Australia south of latitude 26° South to the Middle East from May to October (DAFF 2011). Second, this study highlights the strong correlation between weather conditions (temperature and wind speed) at the departure ports and sheep mortality during sea export. This suggests that the influence of weather at the departure ports should be considered in sheep mortality prediction models, such as the

Australian Heat Stress Risk Assessment model, which is used in Australia to predict the heat-stress-related sheep mortality before export. Third, the key correlates of sheep mortality identified in this study suggest exporters could reduce the mortality risks through either preferentially exporting sheep in the Australian summer or autumn whenever possible, together with shortening the voyage duration and loading sheep at multiple ports. In particular, compared with other departure seasons, export from Australia in the southern hemisphere autumn could significantly decrease sheep mortality rate, which was interpreted by the seasonal variation in pasture availability and tissue mobilization patterns, as well as photoperiod history sheep experienced prior to the export. To investigate sheep mortality during live export thoroughly, future research is required to address the possible contribution of these factors.

Inclusion of humidity at the destination ports in the restricted model highlights the effect of humidity in the Gulf region on the mortality of sheep exported from Australia to the Middle East. Destination humidity was not included in the full model. This may be because humidity at the destination ports is highly variable, which was not captured accurately by the full dataset based on 71 voyage records with actual departure and arrival dates and 346 voyage records with estimated departure and arrival dates. Thus, future research is required to fully investigate the contribution of destination humidity to the sheep mortality during export, based on a large dataset of voyage records with actual departure and arrival dates.

Temperature at the destination ports was not included in either the full model or the restricted model in this study. Although the impact of forecast bias was investigated, the absolute forecast error was determined by an error in 10-day mean air temperature forecasts in Russia (Vil'fand, *et al.* 2010), which did not take into account the potential differences in this error between Middle East and Russia. Additionally, this adjustment may overestimate the forecast accuracy for voyages lasting for more than 10 days, since the Global Forecast System showed that 11-15 days forecasts have a 30% greater absolute errors than the 6-10 days forecasts (WDT 2018). For the shipments with multiple destination ports, weather data of the largest importing country that year was used due to a lack of discharge details. This estimation did not consider the major weather differences between different destination ports, particularly humidity differences, which could be included in future investigation, for which adjusted mortality rate corrected for the multiple discharge ports may be necessary.

Voyage duration, identified as an important correlate of sheep mortality in this study, only included the days from the first sheep loaded until the last sheep was unloaded. If taking into account the mortalities when sheep were transported from the farms to the ship or between discharge and

eventual slaughter (the latter estimated as 3%, Scharp 1992), the effects observed might be enhanced. Time between discharge and slaughter could also be built into the model, but data would be scarce. Thus, adjusted mortality rate corrected for the voyage duration may be necessary for further mortality investigation.

The full model and the restricted model also imply the key role that weather en route plays in the prediction of mortality risks during Australian sheep export by sea. However, due to a lack of both this weather data and detailed voyage records, this study could not take into account the climatic influence en route on sheep mortality. Thus, based on the present study, future research is required to optimize the model fit for sheep mortality prediction by considering the climatic influence en route during sea export and the effects of climate change.

This study suggested future in-depth research is required to address the possible contribution of other factors, such as climatic conditions en route, weather differences between multiple destination ports (particularly humidity differences), climatic conditions at the destination ports or en route, and climate change. Using appropriate statistical models and validation techniques in these future studies will be an essential component of success (Banhazi, *et al.* 2010; Breiman 2001; Chen & Chen 1999).

5.3 Development of a sampling strategy for environmental monitoring on ships

Ammonia accumulation, as one of the top five welfare issues during live sheep export from Australia to the Middle East (Pines, *et al.* 2007), reduced sheep feed intake with adverse effects on chewing activities during eating and rumination, as demonstrated in Chapter 2. However, so far, there is no regulation requiring ammonia monitoring on board shipment in the Australian Standards for the Export of Livestock (DAFF 2011). This is the first study (Chapter 4) to develop an effective sampling strategy for ammonia measurement on an entire vessel or open decks, as well as between or within sheep pens. Taking into account the measurable influence of ammonia exposure in sheep, these sampling strategies are of important value for future regulation to preserve sheep welfare during long distance sea transport. On closed decks, ammonia concentration could not be reliably measured with the number of sites sampled in this study, which suggested more sampling sites are required in future research due to the variable distribution of ammonia concentrations.

Heat stress, as another major cause of sheep mortality during live export from Australia to the Middle East (Caulfield, *et al.* 2014), is related to high air temperature and humidity, particularly

when these are encountered whilst vessels travel through tropical and equatorial regions. However, in Australia only one average recording for dry bulb temperature and humidity is currently required for each deck each day (DAFF 2011), which is hard to indicate the maximum daytime temperature and reflect the distribution of temperature and humidity on board shipment. Thus, this study firstly developed an effective sampling strategy for temperature measurement on entire vessel, open or closed decks, as well as between or within sheep pens. These results suggested that modification of temperature regulation by the Australian Standards for the Export of Livestock (DAFF 2011) is necessary. In particular, if taking into account the effects of climate change on the temperature and occurrence of heatwave days during summer in the Arabian Gulf and the date of the annual Eidh al-Addha festival, this study provides important new evidence for the future temperature regulation to identify and effectively manage the heat-stress-related mortality risks in sheep during live export.

Relative humidity could not be reliably measured even with the maximum number of sampling sites in this study, due to the variable distribution on board shipment during sheep export. More sampling sites are required in future research to develop an effective sampling strategy for relative humidity measurement on an entire vessel or separate open or closed decks, as well as between or within sheep pens. Other factors associated with relative humidity should be considered, such as high stocking density of sheep, inadequate ventilation, or the ingress of sea water on lower decks, depending on the vessel structure design and vigilance of the master and crew (Phillips 2008).

This study estimated the number of sampling sites required for effective environmental monitoring on live export vessels, but did not consider a set of inputs (capital cost, time, and labour), which increase with the number of sampling sites. Future research might be needed to investigate the feasibility of applying automatic monitoring of sampling sites using modern sensors technologies for micro-climatic condition measurement on board shipment. These technologies have been suggested as a possible solution to deal with the increased cost, time and labour input requirements associated with the increasing number of sampling sites (Banhazi & Berckmans 2008; Parkin, *et al.* 2007; Banhazi 2009; Clements, *et al.* 2011). Evaluation and efficiency comparison between manual measurement and utilization of electronic monitoring system may be necessary to provide a practical and affordable method for micro-climatic conditions measurements on the shipments during sheep export. Alternatively, due to the contentious nature of the trade, access to live export shipments by scientists is rarely allowed in Australia. Therefore, all of the simulated sampling strategies evaluated in this study were based on two datasets collected on two voyages for live sheep export from Australia to the Middle East. As the selection of initial sampling sites was according to the predicted ammonia concentrations, the outcomes for temperature and relative

humidity measurement in this study should be further tested with site selection based on temperature and relative humidity distribution on live export vessels. In addition, the results of this study could be compared with further research on board shipments with similar conditions. Even for vessels with different structure or design such as new ships with better ventilation systems and management used in recent years, the proposed method was of important value to establishing relevant sampling protocols for micro-climatic condition measurement during voyages.

5.4 Conclusions

1. Under laboratory conditions, a level of 30 ppm (21 mg/m^3) ammonia exposure for 14 days reduced sheep feed intake and lowered chewing rates during eating and rumination, which may indicate an irritation in the buccal cavity. Ammonia exposure caused stress in sheep as indicated by the increased respiratory rate and faecal corticosterone metabolites concentration, as well as by the development of discomfort.

2. It was proven from published records that sheep exported to the Middle East from Australia in the southern hemisphere winter or spring have substantially higher mortality rates than those shipped in the southern hemisphere summer or, especially, autumn. The key correlates of sheep mortality identified suggest exporters could reduce the mortality risk through either preferentially exporting sheep only in the Australian summer or autumn whenever possible, together with shortening the voyage duration and loading sheep at multiple ports.

3. Weather conditions at the departure ports play an important role in sheep mortality prediction before export, which should be considered in sheep mortality prediction models, such as the Australian Heat Stress Risk Assessment model currently used by Australian livestock exporters. This simple refinement avoids the uncertainty of destination port weather predictions.

4. The sampling strategy developed for ammonia and temperature measurement on board shipment potentially contributes to the accuracy of the assessment of the micro-climatic conditions during long-distance voyages, as well as to the future regulation of environmental monitoring to preserve sheep welfare during live export by sea. The relevant methodology is a positive contribution to the establishment of sampling protocols for environmental monitoring on other vessels, even those with different structure or design.

5. This research project suggests a modification that is required for the current Australian Standards for the Export of Livestock, as there is currently no maximum ammonia concentration proposed for live sheep export and regulation of the environmental monitoring on board shipments. The current regulation on temperature and humidity monitoring during voyages is insufficient, in particular to assess the heat-stress-related mortality risks.

5.5 Further research

Through this project, several related areas of research interest have been identified:

A. Evaluation of the effects of ammonia exposure on buccal cavity health, taste function, and ruminal digestion in sheep with the same ammonia treatment level and exposure period.

B. Evaluation of the effects of ammonia exposure on emotional reactions and relevant physiological responses in sheep with the same ammonia treatment level and exposure period.

C. Assessment of the acute and chronic stress responses of sheep to ammonia exposure during live sheep export.

D. Validating the influence of ammonia exposure on feed intake and nutritional behaviours in sheep through on board research during live export.

E. Optimizing current models for sheep mortality prediction before sea export by including the climatic influence en route and the effects of climate change.

F. Evaluation of the contribution of heat stress, seasonal variation in pasture availability, tissue mobilization patterns, and photoperiod history to the seasonal mortality pattern during live sheep export.

G. Development of sampling strategy for ammonia measurement on closed decks and relative humidity measurement on board shipment during live sheep export from Australia to the Middle East utilizing the methodology proposed and validated for ammonia and temperature measurement.

H. Assessment of the feasibility of utilizing an electronic monitoring system for micro-climatic conditions measurements on board shipment during live sheep export.

6 Chapter 6: List of References

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Appendix-1

THE UNIVERSITY OF QUEENSLAND
AUSTRALIA

UQ Research and Innovation
Director, Research Management Office
Nicole Thompson

Animal Ethics Approval Certificate

25-Mar-2015

Please check all details below and inform the Animal Welfare Unit within 10 working days if anything is incorrect.

Activity Details

Chief Investigator:	Professor Clive Phillips
Title:	Physiological and behavioral responses of sheep to gaseous ammonia
AEC Approval Number:	CAWE/242/14
Previous AEC Number:	
Approval Duration:	24-Sep-2014 to 24-Sep-2015
Funding Body:	Centre for Animal Welfare & Ethics
Group:	Production and Companion Animal
Other Staff/Students:	John Gaughan, Elodie Sadowski, Megan Sullivan, Yu Zhang, Beverley Hutchinson, Andrew Kelly, Milou Dekkers, Laureline Guinnefollau, Gauthier Duval, Thibaut Heron
Location(s):	Gatton Bldg 360 - CAAS

Summary

Subspecies	Strain	Class	Gender	Source	Approved	Remaining
Sheep		Juvenile / Weaners / Pouch animal	Unknown	UQ	12	12

Permits

Provisos

Reporting Proviso:
The CI is required to report any adverse events immediately and to report outcomes and welfare implications found in animals promptly upon completion of study.

Approval Details

Description	Amount	Balance
Sheep (Unknown, Juvenile / Weaners / Pouch animal, UQ)		
17 Sep 2014 Initial approval	12	12

Please note the animal numbers supplied on this certificate are the total allocated for the approval duration

Please use this Approval Number:

1.When ordering animals from Animal Breeding Houses
2.For labelling of all animal cages or holding areas. In addition please include on the label, Chief Investigator's name and contact phone number.
3.When you need to communicate with this office about the project.

It is a condition of this approval that all project animal details be made available to Animal House OIC.
(UAEC Ruling 14/12/2001)

The Chief Investigator takes responsibility for ensuring all legislative, regulatory and compliance objectives are satisfied for this project.

This certificate supercedes all preceeding certificates for this project (i.e. those certificates dated before 25-Mar-2015)